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Microbial techniques for enhanced oil recovery and paraffin removal in wells of heavy oil fields

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(Banwari Lal, Nirmala Bardiya, and N Ramesha)

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An idea of the potential significance of methods aimed at the extraction of extra crude oil in place using enhanced oil recovery process can be gained from the figures presented by Geffen (1976). In the estimates given, the oil recoverable in 1982, was 9% of total oil in the reserves, which is equivalent to about seven times US consumption while 40% was unrecoverable. The world oil fields contain roughly 1.2×10^{12} barrels of oil, which is not recoverable by conventional techniques.

Engineers have been working for many years on chemical and physical methods of enhanced oil recovery (EOR). So far the applications are limited to specially favourable circumstances and the results are often disappointing in terms of extra oil recovered and of the economics of the process (Springham, 1984). A little or less established recovery process than the chemical and physical methods, the microbial enhanced oil recovery process (MEOR) where microbes are deliberately injected into the oil-bearing rock to release oil has special advantages in terms of operating costs and improved efficiency which may make them specially attractive in the long run (Moses and Springham, 1982). They gave a simplified account on the conventional methods of oil recovery. Detailed account on non-biological methods for enhanced oil recovery was given by many good reviews (Groszek, 1977, Van Poolen, 1980, and Shah, 1981). The methods accordingly fall into four classes, thermal methods, miscible displacement, chemical flooding and selective plugging. In thermal method injecting hot water, or steam or other gases by conducting combustion *in situ* in oil well raises the temperature of the reservoir or gas and thus helps in reducing the oil viscosity and facilitate easy oil extraction. The miscible displacement method involves injection of fluids that are miscible with oil reducing the problems of water flooding. Carbon dioxide is used as it dissolves in oil and the swelling caused by that reduces the viscosity. CO₂ flooding is a very attractive measure but large quantity of CO₂ is required and it may range from 112 to 280 m³ per barrel of oil (Meyer, 1977; Shah and Wittmeyer, 1978). In chemical flooding, chemicals are injecting with the water flood which improves the displacement efficiency. A viscous solution of polyacrylamide, a polysaccharide is injected to increase the viscosity of displacing liquid thus improving the sweep efficiency. In fact polymer and micellar flooding

work well on laboratory scale and the success undoubtedly relies on the selection of right reservoir and right treatment when it is applied in the field. The selective plugging is carried out using a viscous polymer or time setting gels into the thief zones and thereby facilitating the flood water to access less permeable regions which would have received only minor amounts of polymer. These EOR processes are becoming more and more attractive as and when they are better understood.

The first suggestion that bacteria might have a useful role in enhanced oil recovery came from Beckman, 1926. Chang (1987) and Adkins *et al.* (1992) conducted laboratory experiments using core samples and sand packed columns and *C.acetobutylicum*, *B. subtilis*, *B. licheniformis* JF-2, *Vibrio aspartigenicus* GSP-1 etc. for MEOR process studies. One of the early field studies on MEOR involved an unconsolidated water flooded sand stone reservoir in union county, Arkansas, USA (Yarbrough and Coty, 1983, Hitzman, 1983). Molasses was injected along with an inoculum of *Clostridium acetobutylicum* into the reservoir and the CO₂ production of oil increased by 250% (Tanner *et al.*, 1991). Reports of successful MEOR use in carbonaceous reservoir have been observed with injection of molasses, and anaerobic acid and gas producing microbes, which resulted in increased oil recovery. Acid and gas production is believed to be important factors in anaerobic oil recovery (Tanner *et al.*, 1991). Other field trials in carbonate reservoirs produced an increase of 60-126% in oil production in Hungary (Hitzman 1983) and 200% increase in Germany (Wagner, 1991). Similarly injection of *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *Bacillus licheniformis* through the oil well casing followed by incubation resulted in an increased oil extraction in China (Zhang and Zhang, 1993). A carefully controlled field study of MEOR at Alton Field in Queensland, Australia, resulted in 40% increase in oil production (Sheehy, 1990). In Russia, nutrient injection into the oil well increased the output of oil by 32.9% (Ivanov *et al.*, 1993).

Microbial enhanced oil recovery (MEOR) can basically be divided into two categories (i) *In situ* microbial techniques and (ii) Injection of microbial products in petroleum reservoirs.

***In situ* microbial technique**

This technique involves the injection of either nutrients or microbial cultures directly into the reservoir where, through the growth of already present or added microbial systems there lead to the production of gas and other metabolites which help in the recovery of oil. This is brought about because of the change in the properties of oil, and selectively plugging of high permeability zones which increases head pressure and thus enhances recovery of oil.

Among the microbial systems present, bacteria are good candidates for MEOR processes because of small size. Yeast and filamentous fungi block the reservoir pore throats and cease the flow of oil from reservoir lithology. The physical conditions present in the reservoirs are (i) high temperatures in the range of 40°C to 120°C (2) anaerobiosis i.e. lack of oxygen (3) salinity because of primary flooding and also due to the inherent rock property (4) pressure in the range of 50-500 atm and (5) carbon substrate such as molasses, whey or starch and starch hydrolysate.

Thus there is a need for thermophilic, barophilic, halophilic, anaerobic bacterial isolates for MEOR processes. Most commonly used micro-organisms for MEOR are given in Table 1. Spore formers *Bacillus* and *Clostridium* sp. have great potential for survival in the reservoirs.

Many anaerobic bacteria produce acids that may result in dissolution of reservoir rock carbonate (Yarbrough and Coty, 1983) and changes visible to the naked eye can be produced in calcite and dolomite cores by bacterial action (Bubela, 1983). Johnson (1979) exploited the property of production of acids resulting in pore enlargement where the vital region immediately round the injection well has been partly physical. Yarbrough and Coty (1983) reported that *Clostridium acetobutylicum* generate pressure upto 8.8 MPa in laboratory culture in the absence of gas phase mainly due to production of hydrogen which has a very low solubility in water. Depressurisation by the production of CO₂, hydrogen, methane and nitrogen was studied. The solution of gases in oil results in swelling and decreased viscosity. Crawford (1983) reported that the plugging of the formation at the vicinity of the well where for geometrical reason large volumes of fluid would pass through a very small cross sectional area of rock, would be particularly detrimental, whereas the beneficial efforts of bacterial growth and metabolism would be most effective further into the reservoir.

Table 1 Microorganism associated with MEOR

Name	Products
A) Aerobic	
<i>Xanthomonas</i>	Polymer
B) Facultative anaerobes	Acids surfactants, polymers, alcohol's
<i>Bacillus, Pseudomonas</i>	
<i>Arthrobacter, Enterobacter</i>	
C) Anaerobic	Gases, acids, alcohol's
<i>Clostridium</i> sp.	
<i>Desulfovibrio</i> sp.	

Injection of microbial products

In addition surfactants are also used for enhancing the oil recovery. But little work has been done on the *in situ* production of biosurfactants by the microbial cells.

Biosurfactants

Biosurfactants are a heterogeneous group of surface active compounds that are produced by micro-organism and are gaining prominence and have already been used for a number of important industrial uses due to their advantages of biodegradability, production on renewable resources and functionality under extreme conditions particularly those pertaining to enhanced crude oil recovery.

Biosurfactants have a tendency to absorb at the interface and bring down the surface tension of the liquids i.e. compounds having surface-active properties of reduction in interfacial tension (IFT) between oil and water. Biosurfactants, produced as metabolic by-products, are not only potentially as effective but offer some distinct advantages over the highly used synthetic surfactants. These include biodegradability and reduced toxicity. They essentially consist of hydrophobic molecules i.e. chains of fatty acids and hydrophilic molecules which include a wide range of organic functional groups such as phosphate containing phospholipids, carbohydrates, esters and alcohol's (Table 2). The mechanism of action of biosurfactants is basically emulsification, reduction in surface tension and rock wetting.

Table 2 Major types of biosurfactants

Biosurfactant types	
Lipopeptides and lipoproteins	Phospholipids and fatty acids
Gramicidins	Phospholipids
Polymyxins	Glycolipids
Ornithine-lipid	Trehalose mycolates
Cerilipin	Trehalose esters
Lysine-lipid	Mycolates of mono, di and trisaccharide
Surfactin, subtilysin	Rhamnolipids
Peptide-lipid	Sophorolipids

Biosurfactants reduce surface tension, critical micelle concentration and interfacial tension both in aqueous solutions and hydrocarbon mixtures. These properties create micro-emulsion in which micelle formation occurs where hydrocarbon can solubilize in water or water in hydrocarbons. Singer *et al.* (1983) isolated an aerobe (H13), which reduced the viscosity as much as 98%, and part of reduction was due

to the formation of an oil water emulsion under the influence of a glycolipid surfactant. Zhang and Quin (1983) obtained similar results. The properties of the various biosurfactants has been extensively reviewed (Cooper, 1986, Haferburg *et al.*, 1986; Fiechter, 1992. Kosaric, 1993, Banat, 1995). MEOR offers major advantages over conventional EOR in that lower capital and chemical/energy costs are required (Sarker *et al.*, 1989). It has been estimated that 50-70% of oil wells in the USA could support microbial growth with pH values from 4 to 8, temperature < 75°C and salinity <10% (Clark *et al.*, 1981). Conditions, which promote the biosurfactant production, were determined for several cultures (Mulligan *et al.*, 1989). The recovery of biosurfactants from the growth medium or microorganism may be desirable, by methanol precipitation or chloroform/methanol extraction after acidification, isopropanol precipitation, acetone extraction etc. (Sutton, 1992; Bryant, 1990). Laboratory synthesis and modification of biosurfactant have been carried out to increase the interfacial lowering and wetting action (Ishigami *et al.*, 1993). A valuable body of experience concerning the selection and strain improvement of suitable organisms is available (Southerland, 1983). These strategies include selection of high yielding mutant, modification of polysaccharide properties and a mutation to eliminate unwanted metabolites. The successful *in situ* polysaccharide/polymer production requires an appropriate nutrient balance. In general for good polysaccharide production, carbohydrate must not be a limiting and high C: N ratio is generally favourable. There are evidences that different nutrient limitations may affect the precise chemical nature of the product (Springham, 1984). Useful survey of some of the complexities that affect interfacial tension such as properties of oil and surfactant, surfactant concentration, salt level, temperature and the nature of co-surfactant have also been provided in the literature.

Biosurfactants are amphiphilic compounds produced by microorganisms which either adhere to cell surfaces or are excreted extracellularly in the growth medium (Fiecher, 1992; Zajie and Steffens, 1984). Many microorganisms produce biosurfactants during growth on a wide variety of substrates. Biosurfactants synthesis by microorganisms has been studied mainly in mesophilic environments using mesophilic microbes and there are very few reports on the production of biosurfactants by thermophilic microorganisms. Among the mesophiles *Bacillus spp*, *Pseudomonas rubescus*, *Agrobacterium tumefaciens*, *Glucobacter cernus* and *Rhodotorula* produce biosurfactant during growth on water-soluble compounds (Haferberg *et al.*, 1986; Tahara *et al.*, 1976; Takahara *et al.* 1978).

Biosurfactant production by thermophilic microbes has not been studied very much. A *Bacillus* strain on a hydrocarbon-containing medium was isolated by Banat (1993) which was capable of producing thermostable biosurfactant. *Bacillus licheniformis* isolated from a petroleum reservoir produced surfactant when grown at 35 - 45°C at 5% salt concentration. Bioemulsifier was also shown to be produced by an extremely thermophilic consortium which was stable at 80°C, 20% salt concentration and over a wide range of pH (Trebbau de Acevado and McInerney, 1996). A strain of *Bacillus subtilis* grown on 2% sucrose at 45°C produced biosurfactant which reduced the surface tension of the medium from 68 dynes cm⁻¹ to 28 dynes cm⁻¹. The biosurfactant retained the surface-active properties after heating at 100°C for 2 h, at different pH (4.5 -10.5) and also at 4% salt concentration (Makkar and Cameotra, 1997).

The techniques used to study the oil release on a laboratory scale are of critical importance. Most-reservoir rock is water-wettable and the surface is carried with a layer of water even when oil fills most of the pore-space. Oil is held in place by surface forces thus the displacement of oil from oil wet surfaces is likely to be of importance in most reservoirs (Springham, 1984). The laboratory simulation of porous reservoir for displacement studies involves two main devices sand packs and cylindrical rock cores. The only problem is the difficult in obtaining permeability of lower order with sand packs. Bubela (1983) described the use of concrete and cores of sintered alundum for simulating porous core rocks. Oil released from these simulatory experiments is measured volumetrically (Jang and Yen , 1983).

Small cores are used to test commercially the effects of polymers and biosurfactant while the large cores are required to facilitate accurate measurements of released oil and to avoid the possibility of end effects. Microbial activities in oil samples, formation rocks and produced water has been described by a number of authors (Westlake, 1983; Moses and Springham 1982). Jack *et al.* (1983) isolated an anaerobic rod shaped bacterium which grew as discrete cells on sucrose medium and but as chain as glucose/fructose medium. The chain is much more effective in plugging the permeable zones. Bubela (1983) found that rod-shaped organism caused greater plugging than did cocci and plugging are more difficult to shift by the application of pressure. There is a general agreement that the injection of larger volumes of desired bacterial suspensions produces a progressive reduction in permeability (Jenneman *et al.*, 1983). The most severe plugging occurs at or close to the injection place. The greater the concentration of cells injected, the greater the degree of plugging. The reports of field trials generally indicate that bacteria can become distributed fairly rapidly and subject to the

permeability limits and there is no sign so far that penetration is a serious limitation to MEOR (Hitzman, 1983, Jenneman, *et al.*, 1983, Jang *et al.*, 1983). Springham (1984) expressed the opinion that plugging is a mechanism of great potential importance and its usefulness will depend on the degree to which plugging can be confirmed to the more permeable zone.

Objectives

- i) Development of thermophilic and anaerobic bacterial system active at 90°C.
- ii) Development of an aerobic consortium for thermostable and halostable biosurfactant.

The basic strategy adopted during this project work was to steadily acclimatise the microbial consortium to the temperatures that they are required to withstand for the *in situ* application for microbial enhanced oil recovery (MEOR). Therefore for the development of the anaerobic, thermophilic, halophilic and barophilic bacterial consortium, the samples collected from various sources were enriched first at 70°C. Thus, one set of anaerobic bottles after on the spot inoculation with the various samples and immediately after transportation to the laboratory was incubated first at 70°C. The bacterial consortium were enriched at 70°C for two cycles, and then a set of anaerobic bottles each were inoculated from the cultures enriched at 70°C, to enrich and acclimatise the cultures at 80 and 90°C separately while still maintain the 70°C cultures at 70°C for better acclimatisation. After four cycles of enrichment at 80°C, another set of anaerobic bottles were inoculated from the cultures being enriched at 80°C to enrich and acclimatise one additional set of cultures at 90°C in a sequential manner, while still maintaining the 80°C cultures at 80°C for better acclimatisation. The results thus obtained for the development of the anaerobic consortia, particularly with samples from Manikaran showed that the enrichment of the anaerobic consortia at 70 and 80°C increased with increasing enrichment cycles and then stabilized. However the volatile fatty acid production pattern at 90°C was not consistent over nine enrichment cycles. This prompted us to re-evaluate the strategy for enrichment at 90°C by introducing an additional step of incubation temperature of 85°C. This essentially meant that after the consortia had stabilized at 80°C, these consortia were then incubated at 85°C first and only after their stabilization at 85°C, the incubation temperature was increased to 90°C.

At all the temperatures (70, 80, 85, and 90°C), one set of uninoculated bottles were kept as control to monitor any possible thermo-chemical changes taking place due to high temperatures. The duration of each enrichment cycle was kept sufficiently long (15-20 days) to provide micro-organisms with enough regeneration time and thus eliminating the possibility of washout of microflora during the process of enrichment and acclimatisation. This pattern of enrichment allows the bacterial consortium to enrich the most efficient bacterial

strains and thus help in the process of the selection of the best bacterial consortium (Figure 1).

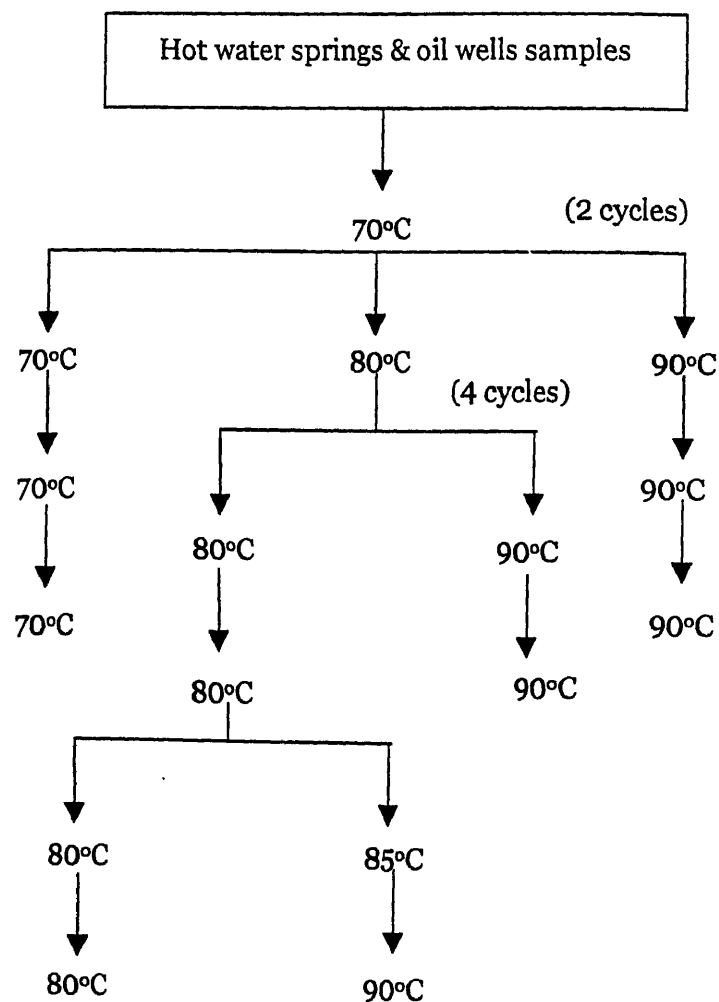
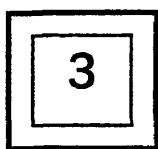


Figure 1. Strategy for enrichment and adaptation of anaerobic microbial consortia

With respect to the development of biosurfactant producing microbes, various nutritional factors influence the production of the biosurfactant. It has been generally observed that the production of the biosurfactant is enhanced under conditions of stress due to non-availability of the carbon source. However, in microbes with constitutive biosurfactant production capability, maximum growth enhances the yield of the biosurfactant. Therefore in this project we had tried both the approaches using two growth media, the basal mineral medium and the rich, Luria broth medium (Appendix) supplemented with 1% crude oil in

both cases. Further the growth temperatures of 55 and 70°C have been used and the thermotolerance of the biosurfactant produced by the various consortia were tested at 70, 80, and 90°C. The microbes are not grown at higher temperatures above 70°C as the growth rates are very low and further the aim was to select the most thermotolerant biosurfactant being produced by the thermophilic microbes.



Methodologies

Development of thermophilic and anaerobic bacterial system active at 90°C

Sample collection for development of anaerobic, thermophilic, halophilic, and barophilic bacterial consortia

Samples for the development of anaerobic, thermophilic, halophilic, and barophilic bacterial consortia were collected from natural habitats such as hot water springs at Manikaran, H.P., Konkan region of Maharashtra, Tattapani, Shimla, H.P., and from oil wells in the Mehsana region of Gujarat. The samples from the oil wells from the Mehsana region were collected from one of the ports using sterile disposable syringes while from hot water springs samples were collected following the standard anaerobic sample collection techniques using a fabricated bottom-sampling device. The sampling procedure as described by Zeikus and Winfrey (1976) was followed for sample collection. The bottom sampler was gassed with purified nitrogen gas to remove the oxygen. The sampler was then dropped into the water and allowed to impact with the sediments, collecting some quantity of mud. The sampler was retrieved and the sediments were transferred into 25-50 ml sterile, pre-gassed, 100 ml serum/anaerobic bottles while being continuously gassed with oxygen free nitrogen and sealed with a butyl rubber stoppers and aluminium caps to maintain anaerobic conditions during transport to the laboratory. The collected samples were used as inoculum @ 10% (v/v) for on the spot inoculation on media (Appendix) prescribed by Balch *et al.* (1977) and Braun *et al.* (1979) for acetogenic bacteria with the help of sterile disposable syringes, a portable N₂ cylinder to maintain anaerobiosis. To lower the redox potential and to maintain anaerobiosis reducing agents @ 0.25 ml/10 ml medium (cysteine -HCl + Na₂S.9H₂O 1.25% w/v solution) were added to the sample (inoculum) bottles at the time of sample collection. The samples were constantly maintained at high temperature in an insulated icebox during transportation to the lab for processing and to incubated at the desired temperatures (70, 80, 85, and 90°C) for enrichment and further screening for selecting the most efficient consortia.

The measurement of temperature of inoculum source was made *in situ* using a thermometer immediately at the time of sample collection.

Anaerobic techniques

Bryant (1972), Miller and Wolin (1974) and Holdeman *et al.* (1977) base the anaerobic techniques followed for enrichment on Hungate (1969) and its modification. All manipulation for anaerobiosis is carried out using a gassing manifold (Biosystems, Pune, India) with N₂.

Preparation of anaerobic liquid media

For preparation of liquid media, all the ingredients of the medium except heat labile and reducing agents were dissolved in boiling distilled water. One ml of 0.1% Resazurin as redox indicator was added to 1 litre medium. The medium was cooled under oxygen free N₂. At this stage, reducing agents were added and pH of the medium was adjusted to 6.8. Completion of anaerobiosis was observed by disappearance of Resazurin colour in the medium. This reduced medium was dispensed in serum bottles pre-gassed with desired gas (nitrogen) in desired quantities. The bottles were sealed with butyl rubber stoppers and aluminium caps using a crimper. The bottles were autoclaved at 121°C for 20 minutes.

Enrichment of the anaerobic bacterial system

All the inocula collected for adaptation at high temperature, enrichment and further screening for selecting the most efficient consortia were incubated at different temperatures - 70, 80, 85, and 90°C. These samples from different sites were maintained in the laboratory under strict anaerobiosis using prescribes standard anaerobic techniques. The samples were constantly maintained at desired high temperatures in incubators for use as seed inoculum during the process of adaptation and enrichment for the subsequent transfers into fresh medium.

Start-up for adaptation and enrichment

The samples collected from different sites were used as basic seed for start-up of the adaptation and enrichment process. Well-mixed inoculum sample was drawn anaerobically in a sterile gas tight syringe. The sample inoculum @ 10% (v/v) was used for inoculation of bacterial population initial at 70°C for two cycles and then separately and sequentially at different temperatures viz., 70, 80, 85, and 90°C as discussed earlier. The adaptation and enrichment of the collected samples was done on media as prescribed by Balch *et al.* (1977) and

Braun et al. (1979) but modified as per requirement for different carbon sources such as glucose and molasses at experimental temperatures. The consortia were constantly maintained at desired high temperatures in incubators during the process of adaptation and enrichment and for use as seed inoculum for the subsequent transfers into fresh medium. At regular time interval, fixed volume of sample was withdrawn for various analyses such as gas composition, volatile fatty acids, carbohydrates, and protein to constantly monitor the adaptation and enrichment process.

Enrichment of thermophilic anaerobic consortia

Well-mixed inoculum sample was drawn anaerobically in a sterile gas tight syringe. The sample @ 10% (v/v) was used for enrichment of bacterial population. The samples brought from the hot water spring sites and from oil wells in the Mehsana region were inoculated into different liquid media of Balch *et al.* (1977) for enrichment of different microbial consortia at 70, 80, 85, and 90°C. The presence of carbon dioxide (analysed by GC) was used as an indicator for enrichment of acidogens. Consortia were transferred for further enrichment into a fresh anaerobic medium when carbon dioxide content ranged between 7–10% (v/v). The presence of carbon dioxide (as analysed by GC) was used as an indicator for enrichment of acidogens.

Product identification during growth of anaerobic bacterial consortium

Gas production rates, composition of gas (CH₄ and CO₂ content), pH of medium, volatile fatty acids profile, total carbohydrates, and total protein were analysed as described:

Gas analysis

Composition of the gas produced during growth was analysed by using a Gas chromatograph (Nucon Series 5765) equipped with Thermal Conductivity Detector (TCD) and a 2 meter Porapak Q (80 to 100 Mesh) column. Hydrogen was used as a carrier gas at a flow rate of 20 ml/min. The oven, injector and detector temperatures were maintained at 60, 80 and 80°C respectively.

pH

pH of the medium during growth of anaerobic bacteria consortium was measured using a calibrated orion pH meter.

Volatile fatty acid profile

Volatile fatty acids (VFA) during different states of growth were estimated by Gas chromatograph equipped with Flame Ionisation Detector (FID) and a 1.25 m long chromosorb 101 (80 to 100 mesh) column. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. The oven, injector and detector temperatures of GC were maintained at 170, 200 and 220°C respectively.

Gram staining of aerobic/anaerobic bacterial cells

The liquid culture was centrifuged and the pellet was resuspended in distilled water and a thin smear of this liquid culture was prepared on a clean slide and fixed by gentle heating. The smear was stained with crystal violet for 1 min and the excess stain was gently washed off. A few drops of mordant (Gram's iodine) was put on the smear and was left for 30 sec. The slide was then washed with 90% ethanol, air-dried and stained with Safranin for 1min. The slide was again washed with water, air-dried and was observed under light microscope at 100 × magnification.

Scanning electron microscopy (SEM) of anaerobic bacterial cells

The liquid culture (20-50 ml) was centrifuged at 8000 rpm and the pellet was resuspended in potassium phosphate (50 mM) buffer (Appendix) and washed 2-3 times using same buffer. The cells were resuspended and fixed in fixer solution (Appendix) and was kept at room temperature for 3 hours. The cells were again resuspended in potassium phosphate (50 mM) buffer and washed 2-3 times. Bacterial cells were scanned using scanning Electron Microscopy, at AIIMS, New Delhi.

Development of an aerobic consortium for thermostable and halostable biosurfactant

Development of thermophilic aerobic bacterial consortia for biosurfactant production

Thermophilic aerobic consortium was developed from sample collected from the hot water springs and from oil wells in the Mehsana region. These cultures were maintained in basal mineral medium as well as Luria broth (Appendix) containing 1% (v/v) crude oil as an inducer for biosurfactant production. The cultures were maintained at 55, 70, 80, and 90°C on a mechanical shaker at 200 rpm. Periodically the cultures were sub-cultured in order to maintain the physiological status of the consortium.

Screening the aerobic consortia for biosurfactant production

In order to screen the biosurfactant producing consortium, from the above samples 4-5% of the inoculum was added to the basal medium and Luria broth respectively each containing 1% (v/v) crude oil and incubated at 55, 70, 80, and 90°C respectively. Samples were drawn from the growing cultures at every 24h interval, centrifuged at 12,000 x g for 10 min using Hitachi refrigerated centrifuge and the supernatant assayed for biosurfactant activity.

Biosurfactant assay

The culture supernatant so obtained after centrifugation was used to screen for the presence of extracellular biosurfactant. An aliquot of the culture supernatant was taken in a glass test tube into which crude oil (1%, v/v) was added. This supernatant-oil mixture was vortexed on a cyclomixer at full speed for 1 min and then allowed to stand for 10 min at room temperature. The supernatant-oil emulsion was carefully removed to record the optical density at 610nm in Hitachi UV-visible spectrophotometer.

Study of thermal stability of the biosurfactant

Application of the biosurfactant would be at high temperature such as 90°C. Hence, it is essential to study the thermal stability of the biosurfactant and screen for such consortium that can produce biosurfactant capable of withstanding and performing at high temperatures. Hence, the culture supernatants were incubated in water baths maintained at 30, 70, 80, and 90°C for 1h. At the end of the incubation, biosurfactant activity was assayed as described earlier.

Isolation of bacterial strains from consortia for producing thermostable biosurfactant

The bacterial consortium Mn-1, Ah-2 and B-1 which showed the maximum production of thermostable biosurfactant were then plated on Luria Agar containing phytigel (1.5%, w/v). A total of 560 colonies were isolated and screened for the production of thermostable biosurfactant at 24 h intervals. The 181 isolates showing the maximum thermostable biosurfactant activity, were then reassessed for the biosurfactant activity and the best 5 isolates were then selected for further studies.

Determination of surface tension and calculation of critical micelle concentration (CMC)

Surface tension of biosurfactant was measured using Surface-tensiometer, at IMTECH, Chandigarh.

Measurement of Surface tension of water and biosurfactant: Instrument was set to zero before the commencement of the study. A 20-50ml of distilled water was taken in a clean beaker. Platinum ring of the instrument was first immersed completely inside the water level. Later slowly, the level of the platinum was adjusted in such a way that the surface of the ring sits above the surface of liquid (in this case it is distilled water.) The surface tension was measured by slowly raising the platinum ring until the liquid film between the liquid and ring breaks. Similarly surface tension of crude biosurfactant (culture supernatant, 96h) was measured.

Measurement of critical micelle concentration of biosurfactant: Different concentration of biosurfactant 10–100 % was prepared using distilled water. The surface tension of each dilution was measured as described above. The critical micelle concentration was calculated by plotting the surface tension of biosurfactant at different concentrations.

Optimization of culture conditions for biosurfactant production

Growth of the bacterium would be too low at higher temperatures in basal mineral medium containing crude oil. Hence, nutrient rich medium was essential to increase the bacterial biomass for production of biosurfactant.

Optimization of culture conditions was performed using the selected four isolates that have shown efficient biosurfactant production. Different carbon sources such as glucose, fructose, sucrose, arabinose, maltose, and mannitol (1%) were used in mineral nutrient medium. In addition, Luria broth (100%) and mineral medium containing 10% Luria broth were also used in the study. Quantity of biosurfactant produced was monitored periodically using spectrophotometer.

Similarly, different nitrogen sources were also prepared in basal mineral medium. Nitrogen sources used for the study include ammonium chloride, ammonium nitrate, potassium nitrate, and urea. Luria broth was used as positive control.

Biosurfactant production was monitored spectrophotometrically. Incubator temperature was set at 30°C, 37°C, 55°C and 70°C to look for optimum production of biosurfactant. Luria broth was used as the medium for the growth

of the bacterium. Biosurfactant production was monitored periodically as explained above.

Chemical characterization of biosurfactant: Protein content

Total bacterial protein was determined by Biuret method (Itzhaki and Gill, 1964) using a standard solution of fraction V of bovine serum albumin (BSA). The volume of protein sample was made to 2 ml and 1 ml of biuret reagent was added. The absorbance was taken after 10 minutes at 310 nm using spectrophotometer.

Bradford (1976) dye binding method was also used for the determination of total bacterial cell protein using BSA as standard protein. The sample volume was made to 100 μ l to which 1 ml of Bradford reagent was added. The reaction mixture was left at room temperature for 5 minutes and the absorbance was measured at 595 nm spectrophotometrically (Hitachi, Japan).

Carbohydrate content

Carbohydrate was measured by anthrone method (Updegraff, 1969) using glucose as standard. The total volume was made to 2 ml with sample and distilled water and 4 ml of anthrone reagent (Appendix) was added. The mouth of the test-tubes were covered with glass-marbles and kept in a boiling water-bath for 16 min. They were cooled at room temperature and the absorbance was read at 620 nm against a reagent blank.

Lipid content

Dried partially purified sample was extracted with chloroform: methanol solution (2:1, v/v). The suspension was allowed to stand at room temperature for 5 min. The sample was centrifuged and supernatant collected. An aliquot (0.2 volume) of distilled water was added to the sample and vortexed. Lower layer containing the lipid was transferred to a fresh tube and the sample was lyophilized and weighed to get the lipid content.

Generation of hyper biosurfactant producing bacterial isolates

Exposing actively growing bacterial culture to short wavelength of ultraviolet rays (UV-254nm) random mutation was induced. The culture was exposed to UV light from a distance of 6 inch. An aliquot was drawn at regular intervals (up to 10 minutes) for studying survival of the culture after exposure. These samples were diluted appropriately using sterile saline and plated on Luria agar plates, incubated at 55°C for development of colonies. Rate of mortality of bacteria

calculated at different time intervals. Time taken to attain 10% survival was taken, as the time required for inducing mutation. Those 10% of the survived cultures were selected for monitoring enhancement in biosurfactant production.

Genetic studies with the hyper biosurfactant producing isolates

The plasmid from the selected isolates was purified by the method explained by Kado and Liu (1981). Overnight culture (grown at 55°C) was centrifuged to separate the cells from the culture medium. The cells thus obtained were washed with phosphate buffer and used for isolation of plasmid. To this, cells were suspended in 0.35 ml sterile distilled water in to which 0.70 ml lysis solution, 50 mM Tris Cl pH 8.0 and NaOH (pH 12.6) were added. This mixture was incubated at 55°C for one hour. A 0.35 ml phenol chloroform (1:1) mixer was added to precipitate all the proteins. The precipitated protein was removed by centrifuging the mixture at 13,000 rpm for 30 min. The plasmid containing supernatant was collected and treated with iso-propanol to precipitate the plasmid DNA. Again this mixture was centrifuged at 13,000 rpm for 30 min, the DNA pellet was air dried to remove traces of alcohol and dissolved in Tris EDTA buffer. The plasmid thus obtained was transformed into *E. coli* that was capable of growing at mesophilic temperature and not producing biosurfactant (Maniatis *et al.* 1982). The *E. coli* containing plasmid was tested for biosurfactant production.

MEOR experiment using sand packed column

Microbial enhanced oil recovery experiment was conducted in sand packed column using stainless steel (SS) cylinder (15 cm x 7 cm) which had three outlets. One was connected to a pressure gauge while the second was connected to the inlet tubing, which was perforated at regular intervals. The third was the outlet for the release of oil. The assembly was placed in a water bath maintained at 90°C. The SS cylinder assembly was filled with sand (100-200 mesh), saturated with water and then Mehsana crude oil was added at 90°C. After two days of equilibration, water was pumped from the inlet point and the water & oil coming out from the outlet was collected. The crude oil was extracted and measured gravimetrically. Subsequently the sand packed column was inoculated with the biosurfactant or selected S-2 consortium grown on glucose or molasses as carbon source at 90°C. After one day of incubation at 90°C in case of biosurfactant study and 20 days with S-2 consortium, the column was pumped with water at 90°C, the water and oil coming out of the column was collected, and crude oil extracted and then measured gravimetrically.

Development of thermophilic and anaerobic bacterial system active at 90°C

The various consortia collected from Manikaran, H.P. were used as inoculum for the development of anaerobic, thermophilic, halophilic, and barophilic bacterial consortia as well as the biosurfactant producing consortia. The characteristics of various samples from Manikaran are shown in Table 3. The anaerobic, thermophilic bacterial consortia were grown on Balch *et al.* (1977) and Braun *et al.* (1979) media at 70°C. No growth was observed on Braun *et al.* (1979) medium while there was very good growth on Balch *et al.* (1977) medium. Therefore, for further studies Balch *et al.* (1977) medium was used for further studies. The anaerobic, thermophilic bacterial consortia were enriched at 70°C for two cycles of 15-20 days each and then used as inoculum to incubate at 70, 80, 85, and 90°C for further enrichment as described earlier. A control (without any inoculum) was kept to check any possible thermo-chemical changes occurring in the media components due to high temperatures.

Table 3 Characteristics of the samples from Manikaran, H.P.

Sample No	Place	Source	Sample Temperature (°C)	Sample texture and Colour
Mn-1	Manikaran	Hot water spring	NA	Blackish soil with plant residue
Mn-2	Manikaran	Hot water spring	NA	Clear water with black-brown sand crystals
Mn-3	Manikaran	Hot water spring	64	Clear water with greenish sand
Mn-4	Manikaran	Hot water spring	65	Clear water with yellowish brown residue
Mn-5	Manikaran	Hot water spring	71	Clear water
Mn-6	Vashist	Hot water sulphur spring	50-60	Water with blackish-brown soil

During the enrichment and adaptation process, the anaerobic, thermophilic bacterial consortium at 70°C were analysed for two parameters viz., the volatile fatty acid (VFA) profile and the gas production when grown on glucose (single and double strength) and molasses (single and double strength). Out of six samples from Manikaran only three showed good growth in terms of VFA and CO₂ production (Figure 2 to 9). Among the three, consortium Mn-1 was the best followed by Mn-6 and then Mn-4 irrespective of the carbon source used for the growth. Only acetic and propionic acids were detected among the volatile fatty

acids as products while among the gases, CO₂ was the only product detected except in few samples in which formation of methane (CH₄) was also observed. Consortium Mn-1 showed upto 680% increase over zero time, in the acetic acid production, the maximum being with single strength molasses (Figure 6). Similarly, again consortium Mn-1 was the best in terms of gas production with CO₂ as the only detectable products when grown on glucose (Figure 3 & 5), but CH₄ along with CO₂ was observed when grown on single strength molasses (Figure 7) while consortium Mn-6 showed CH₄ along with CO₂ production on both single and double strength molasses (Figure 7 & 9).

During the enrichment process at 80°C, again consortium Mn-1, Mn-4, and Mn-6 were the best among all the six consortia (Figure 10 to 17). At 80°C, there was not much product (VFA and gases) formation when these consortia were grown on glucose - both single and double strength (Figure 10 to 12). However, there was VFA (as acetic acid) and CO₂ production by consortia when grown on molasses - both single and double strength (Figure 14 to 17). Consortium Mn-1 and Mn-6 showed enhanced acetic acid and CO₂ production when grown on molasses. Consortium Mn-1 produced approximately 1800 ppm of acetic acid after 360 hours of incubation, an increase of 1206% over the zero time while consortium Mn-6 produced only about 430 ppm, a 138% increase over zero time (Figure 14). Following the trend of VFA formation, consortium Mn-1 showed 25% (v/v) CO₂ production as compared to only 3.5% (v/v) by consortium Mn-6 (Figure 15). When the consortium Mn-1 was grown on molasses (double strength), there was a decrease in the both VFA and CO₂ as compared when it was grown on molasses (single strength). However, consortium Mn-6 showed stimulation in VFA (1015 ppm, an increase of 227% over zero time) and CO₂ (18.69%, v/v) production (Figure 16 & 17) when grown on molasses (double strength).

Among the six consortia from Manikaran, Mn-1, Mn-4 and Mn-6 were the best when grown on molasses- both single and double strength at both 70 and 80°C. Further among these three consortia, Mn-1 and Mn-6 were better than Mn-4 and also molasses double strength showed higher volatile fatty acid production than single strength, therefore, Mn-1 and Mn-6 were selected for further enrichment. Among the two consortia Mn-1 and Mn-6, consortium Mn-6 was the best at both 70 and 80°C and after 14 to 16 cycles of enrichment at both 70 and 80°C, volatile fatty acid production after 310/350 hours stabilized at around 1500 ppm, an increase of around 300% over the zero time volatile fatty acid concentration (Figure 18 & 19).

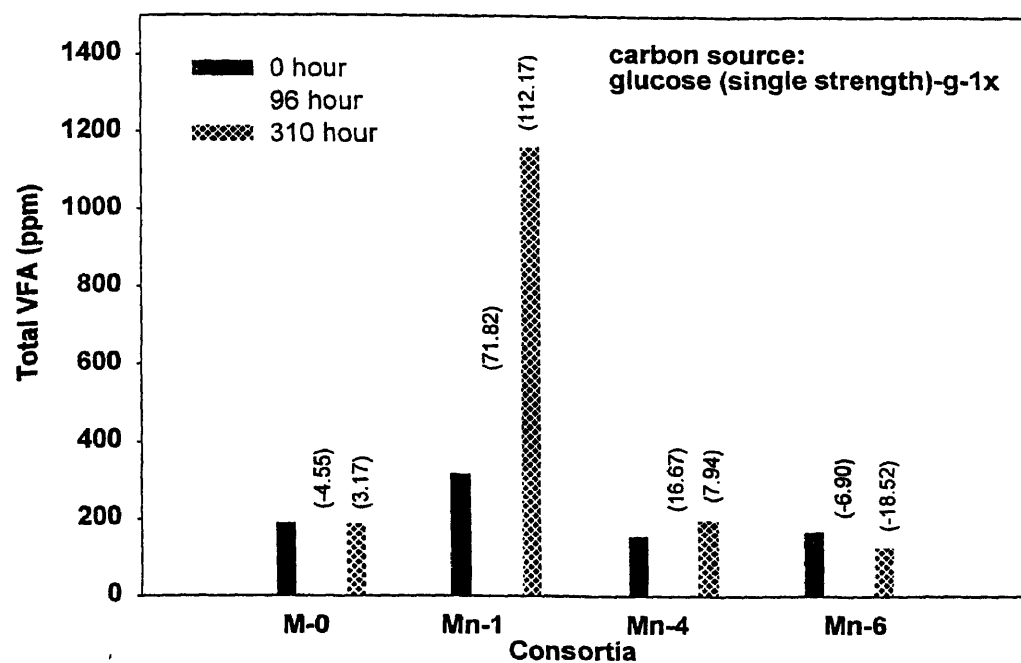


Figure 2. VFA production at 70 °C by the consortia from Manikaran hot spring sites

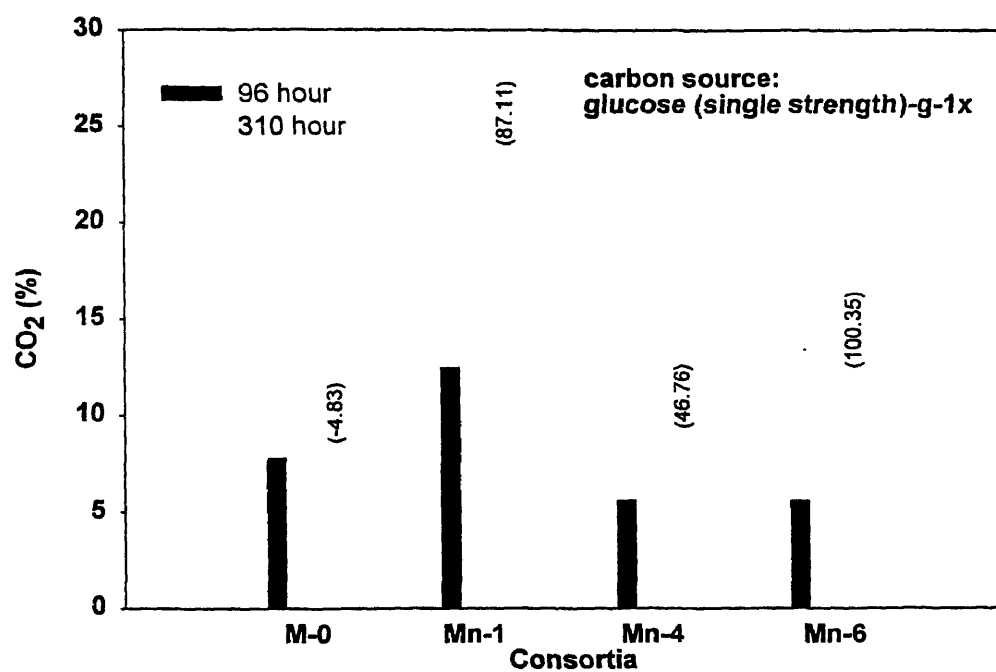


Figure 3. Composition of the gas produced at 70 °C by the consortia from Manikaran hot spring sites

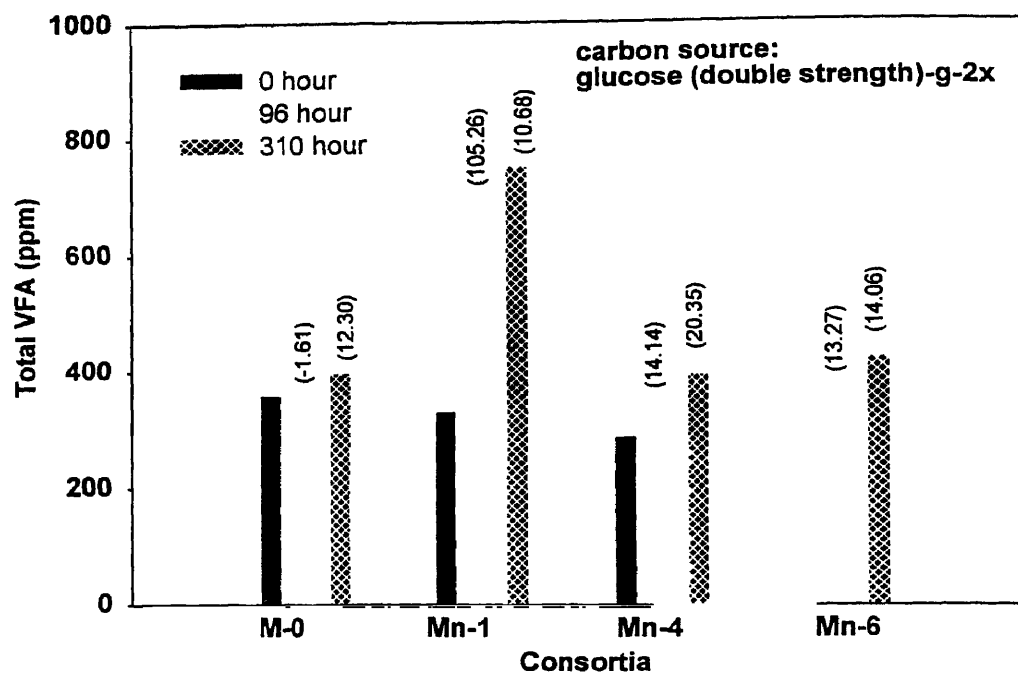


Figure 4. VFA production at 70 °C by the consortia from Manikaran hot spring sites

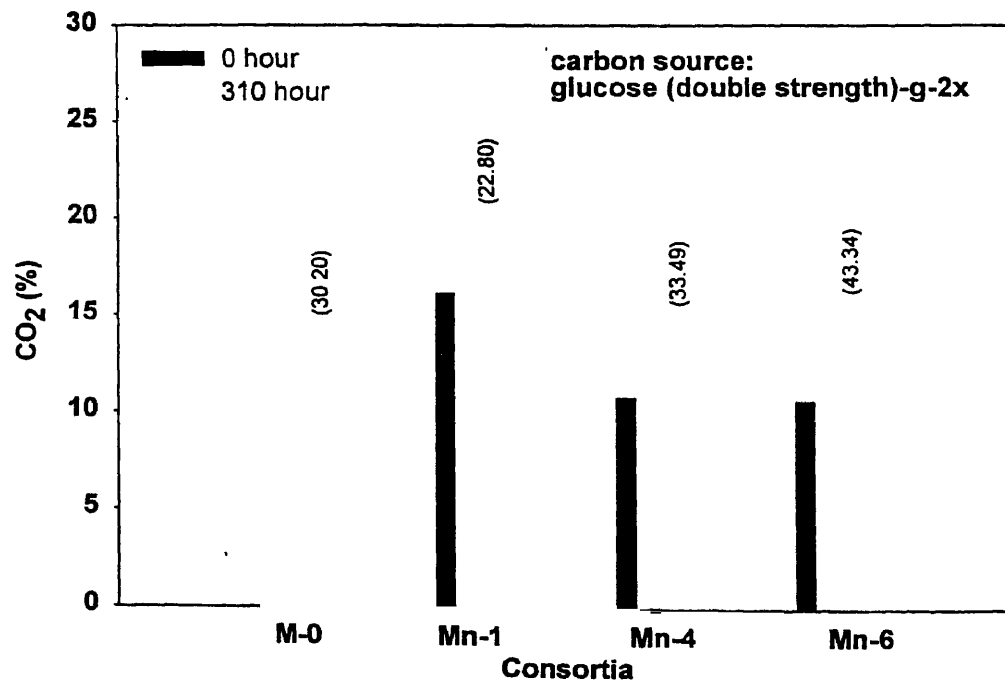


Figure 5. Composition of the gas produced at 70 °C by the consortia from Manikaran hot spring sites

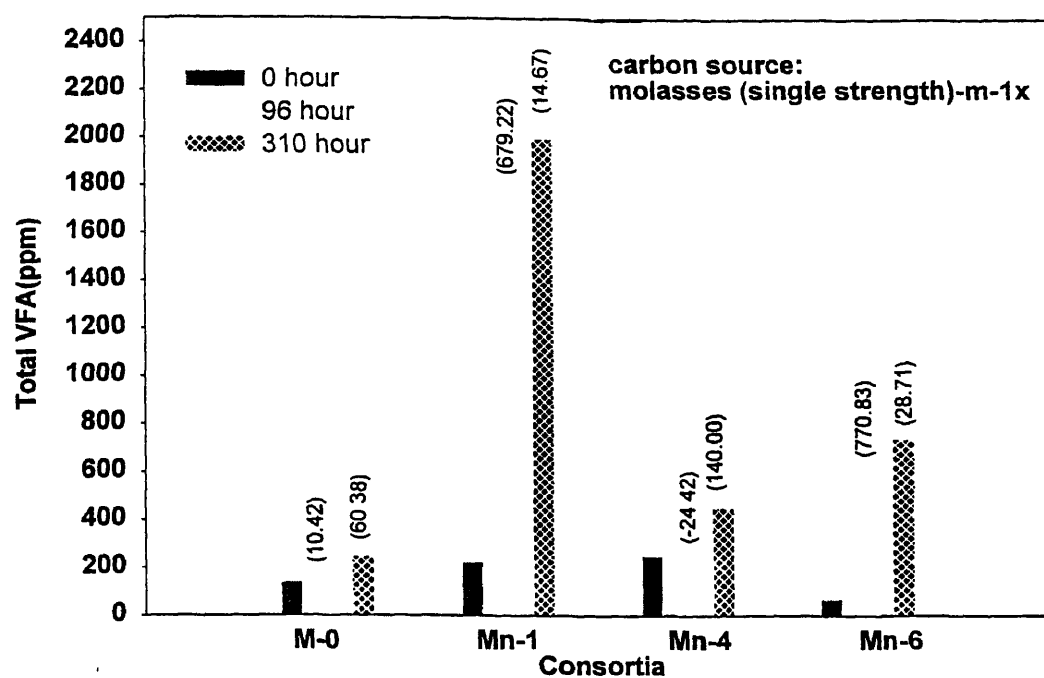


Figure 6. VFA production at 70°C by the consortia from Manikaran hot spring sites

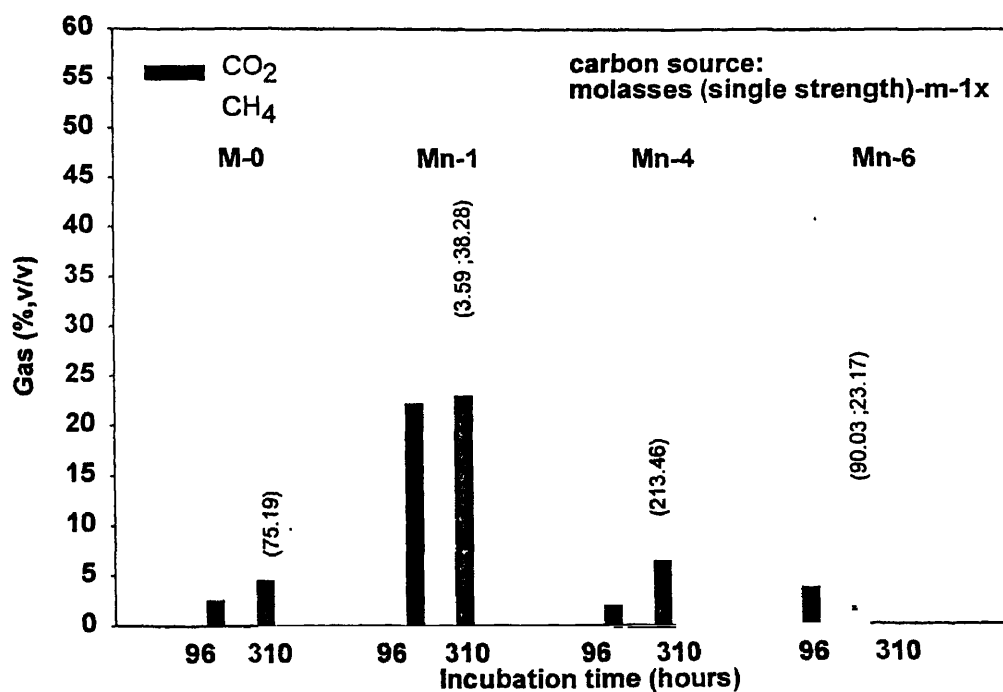


Figure 7. Composition of the gas produced at 70°C by the consortia from Manikaran hot spring sites

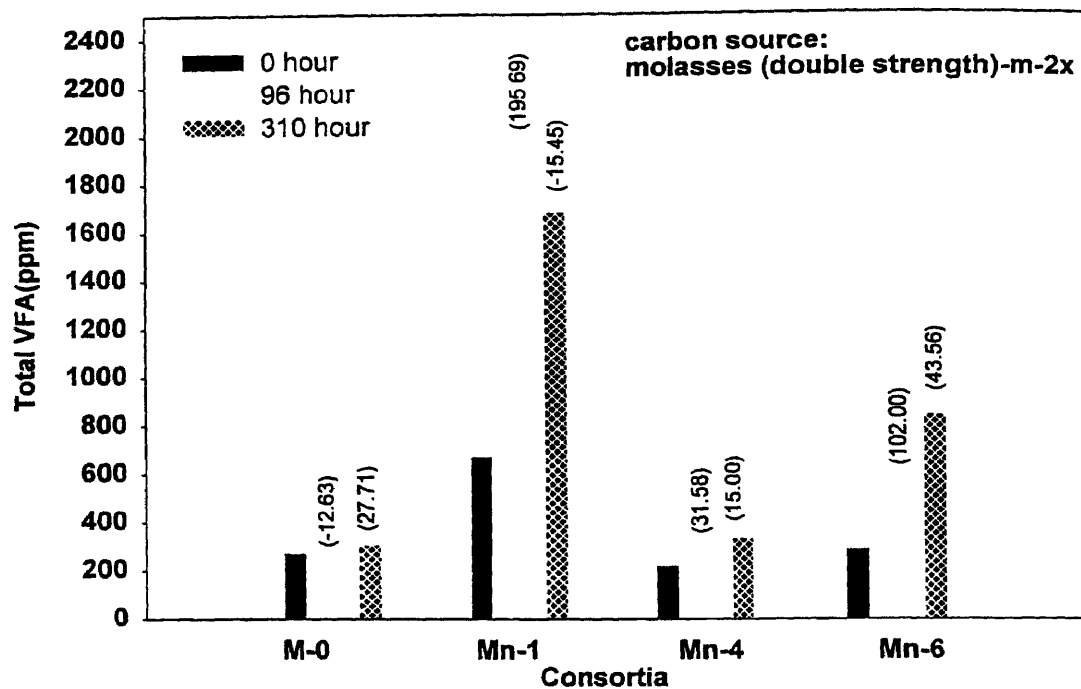


Figure 8. VFA production at 70°C by the consortia from Manikaran hot spring sites

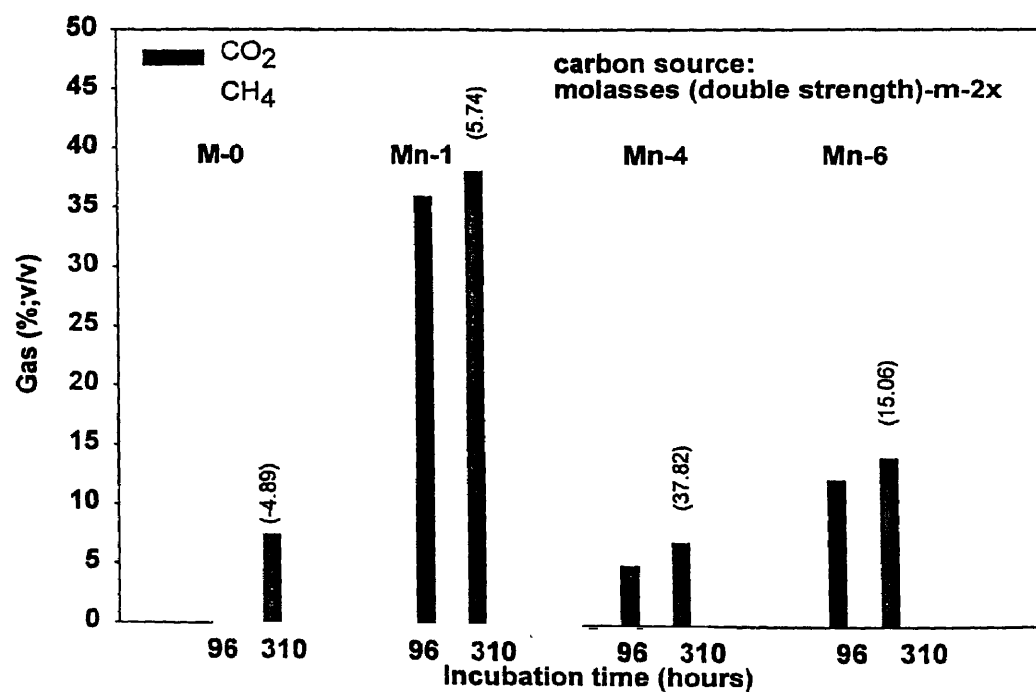


Figure 9. Composition of the gas produced at 70°C by the consortia from Manikaran hot spring sites

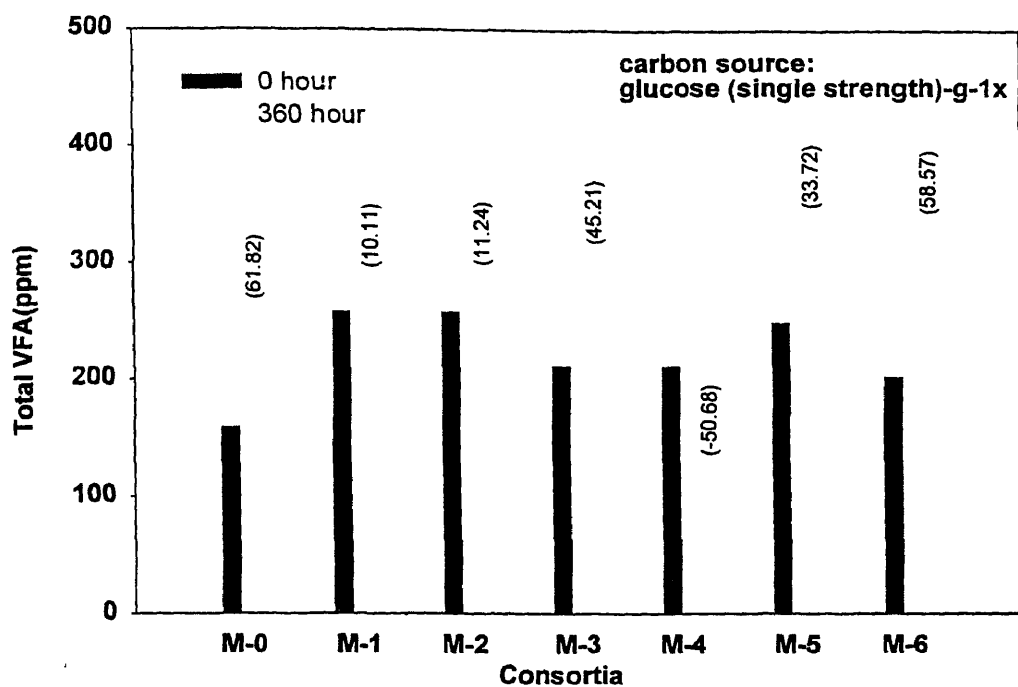


Figure 10. VFA production at 80°C by the consortia from Manikaran hot spring sites

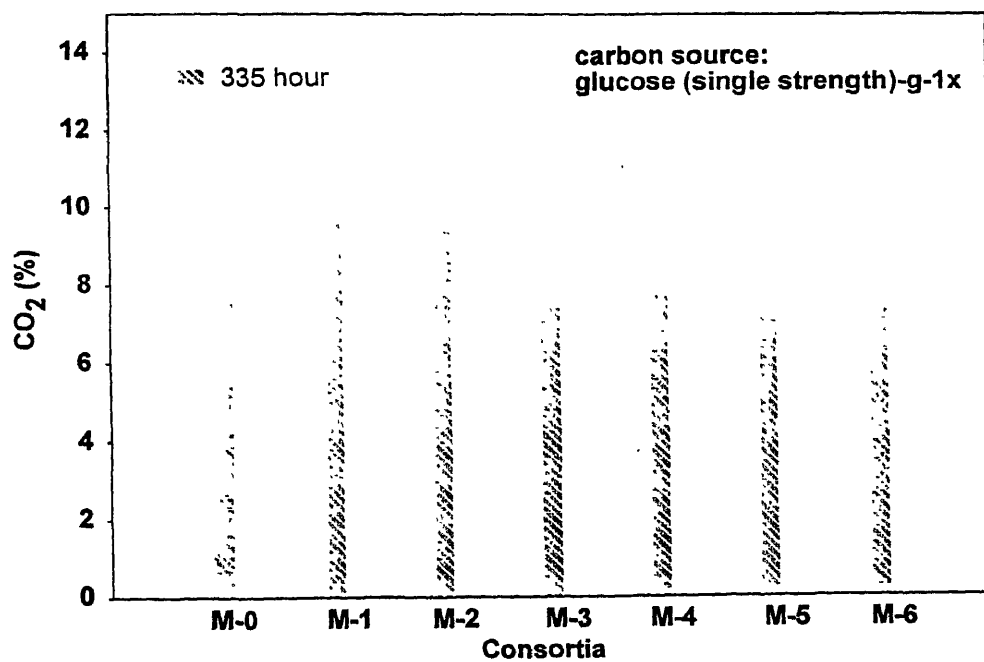


Figure 11. Composition of the gas produced at 80°C by the consortia from Manikaran hot spring sites

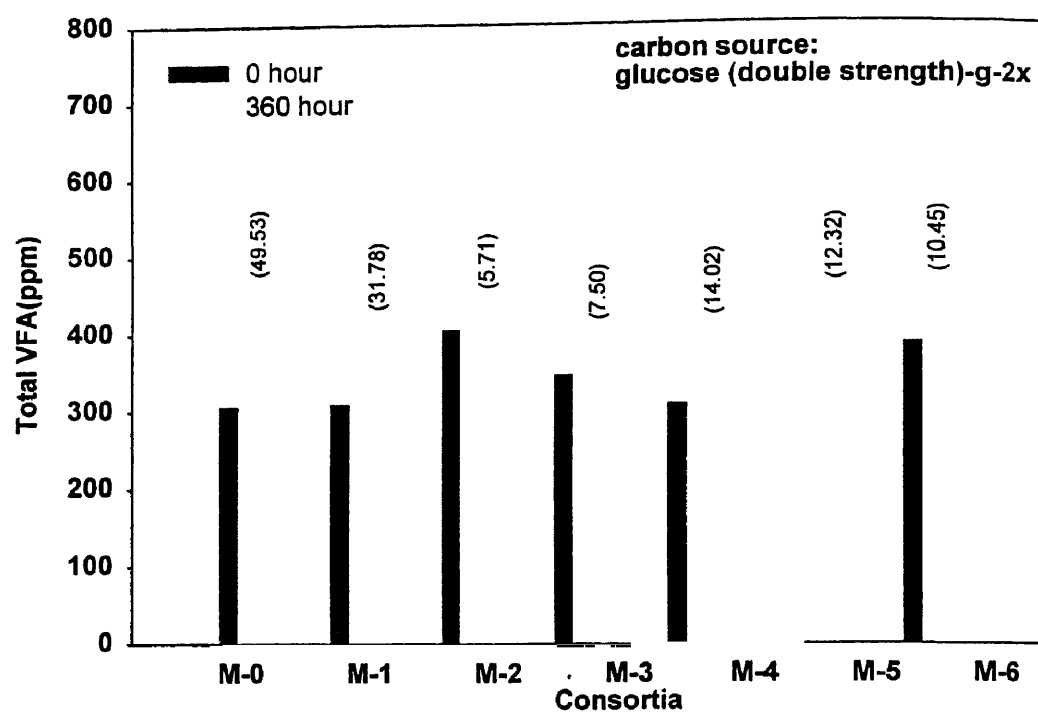


Figure 12. VFA production at 80 °C by the consortia from Manikaran hot spring sites

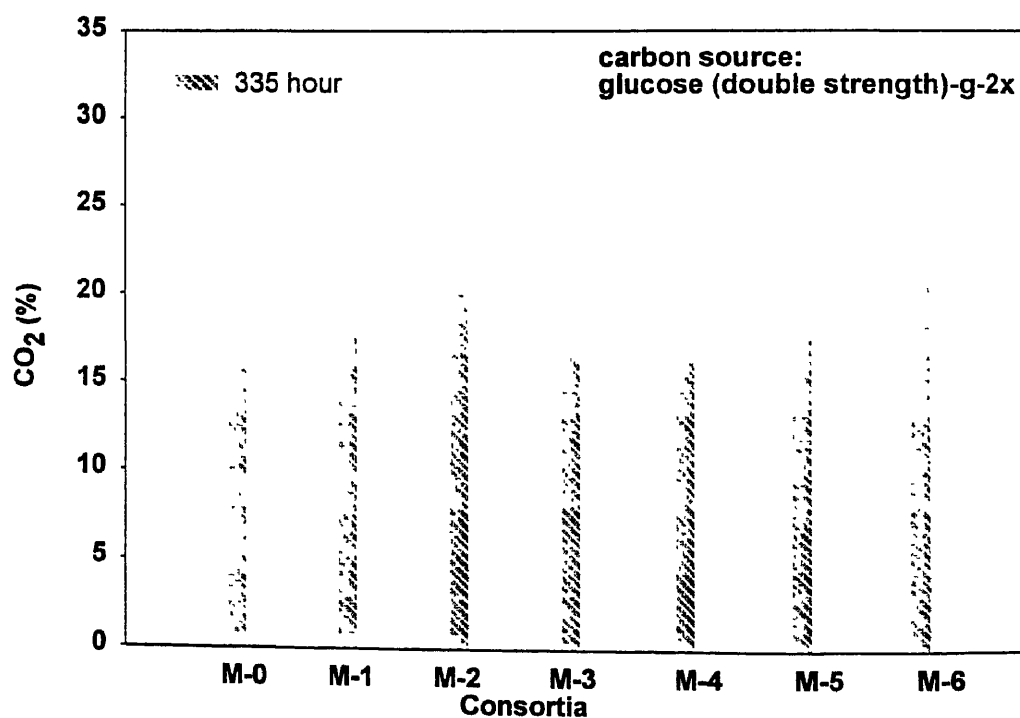


Figure 13. Composition of the gas produced at 80 °C by the consortia from Manikaran hot spring sites

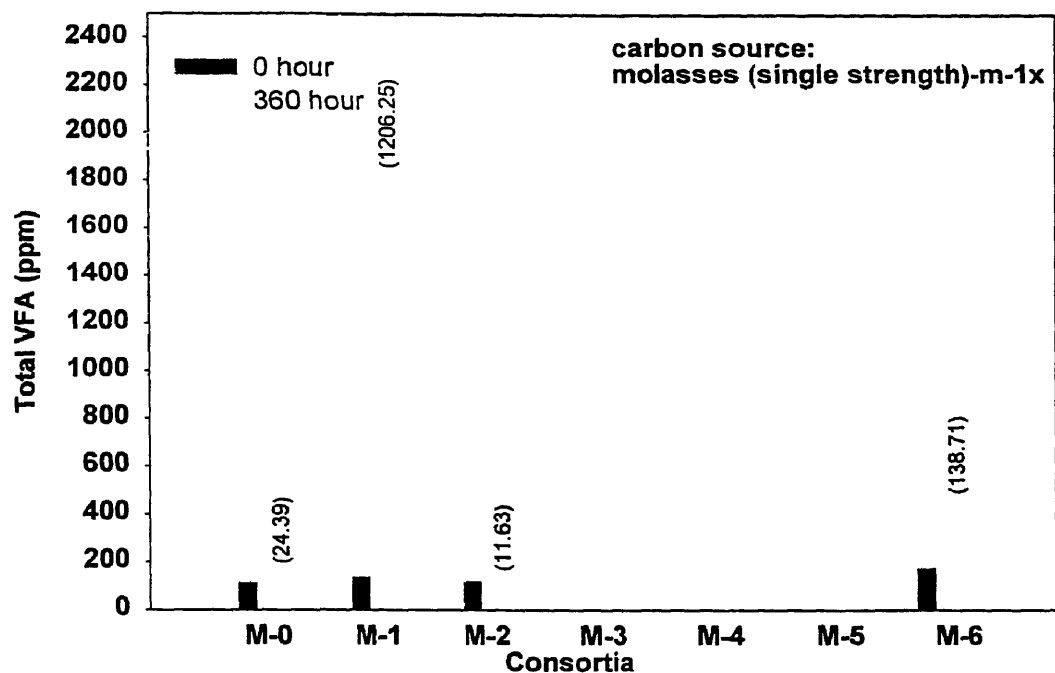


Figure 14. VFA production at 80 °C by the consortia from Manikaran hot spring sites

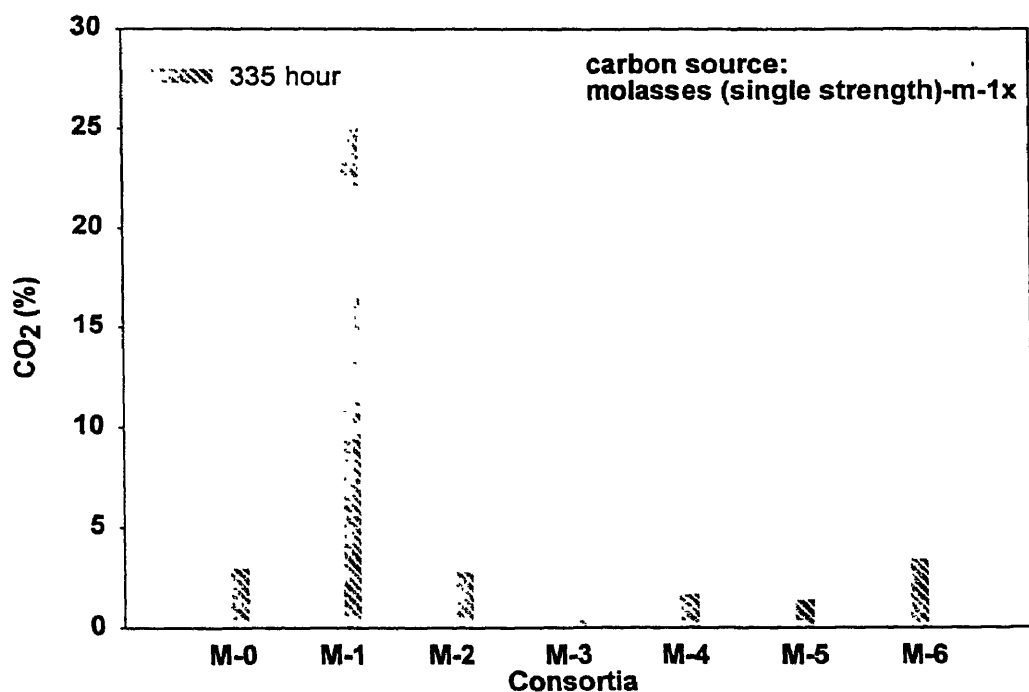


Figure 15. Composition of the gas produced at 80 °C by the consortia from Manikaran hot spring sites

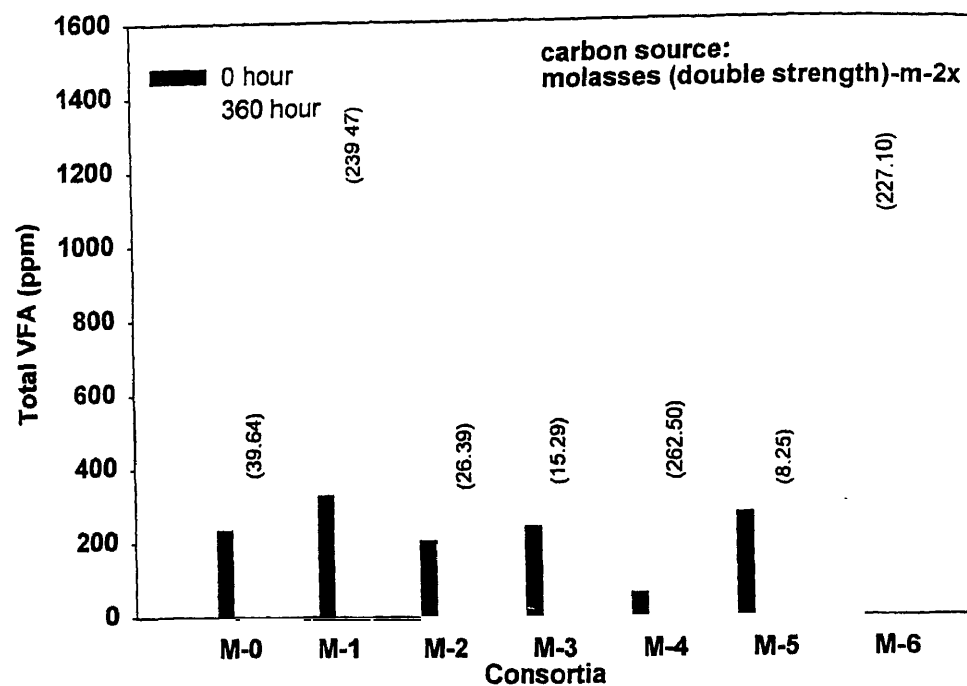


Figure 16. VFA production at 80 °C by the consortia from Manikaran hot spring sites

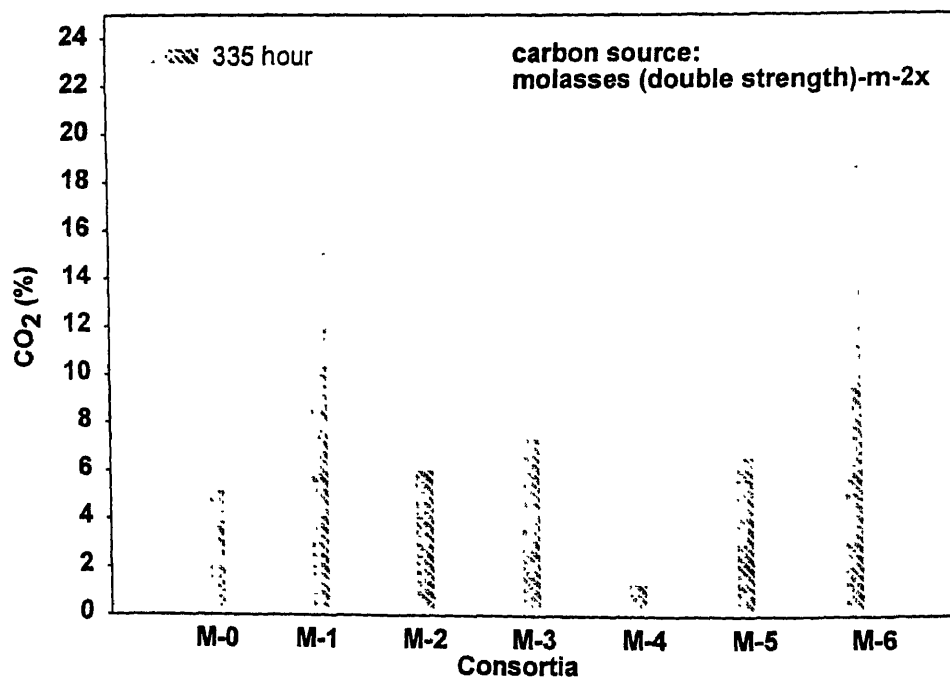


Figure 17. Composition of the gas produced at 80 °C by the consortia from Manikaran hot spring sites

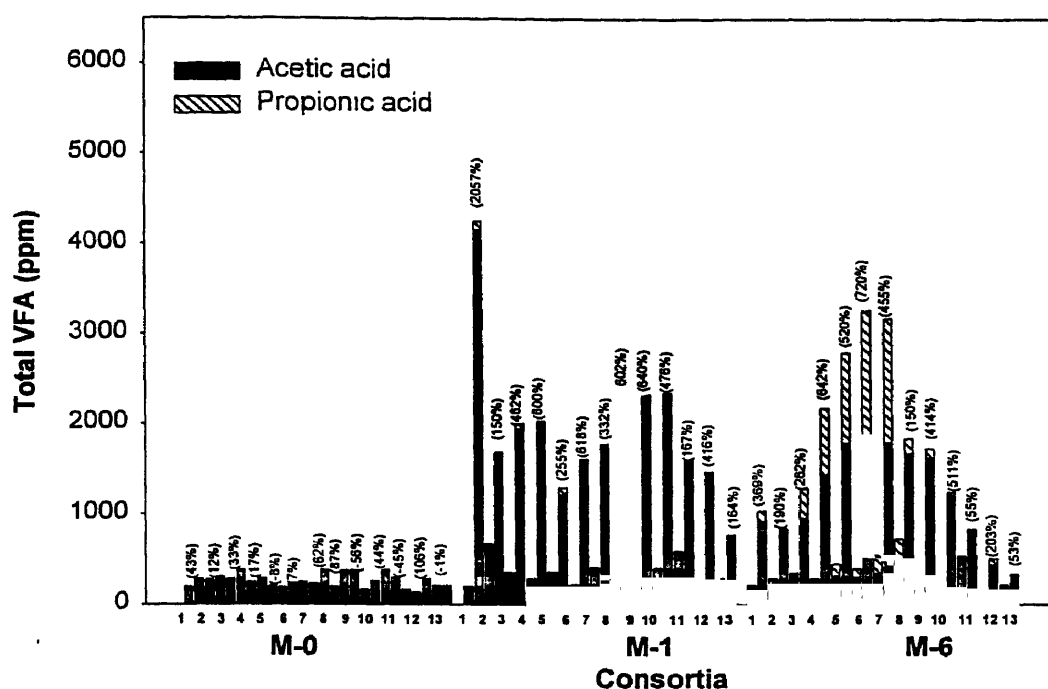


Figure 18. VFA production by selected Manikaran consortia at 70 °C on molasses (double strength) as carbon source during various cycles of enrichment

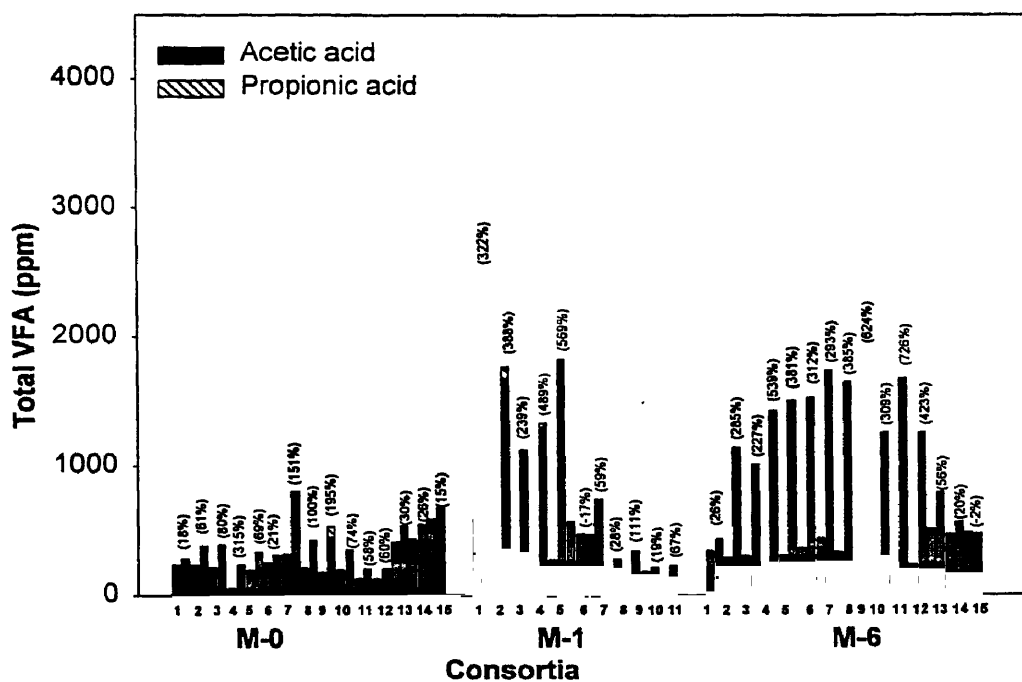


Figure 19. VFA production by selected Manikaran consortia at 80 °C on molasses (double strength) as carbon source during various cycles of enrichment

The consortia Mn-1 and Mn-6 when grown at 90°C, over 16 enrichment cycles showed that volatile fatty acid (VFA) production was not consistent on both glucose and molasses as compared to the control in which no inoculum was added (Figures 20 & 21). In fact we observed thermo-chemical production of volatile fatty acids in the un-inoculated control at 90°C. As mentioned in the strategy, we had introduced two additional steps of enrichment, one at 85°C from 80°C before directly going to 90°C from 80°C and another from 80°C to 90°C before directly going to 90°C from 70°C while the previous sets of enrichment cycles at 90°C from 70°C were continued.

Consortium M-2 and M-5 from Manikaran when grown on molasses (double strength) at 90°C showed enhanced volatile acid production over zero time. The production of volatile fatty acid was not very consistent although over the last two cycles marginal to substantial increase was observed. This indicated that the enrichment of the consortia was continuing to get stabilized (Figure 21).

As a first strategy, during the growth of the six Manikaran consortia at 85°C, only M-6 showed higher VFA production over and above in comparison to the thermo-chemical VFA formation observed (Figure 22) in the un-inoculated control (M-0) with glucose as carbon sources (double strength). However, when the same consortia were grown on molasses (double strength), most of consortia showed much higher VFA formation (Figure 23) over un-inoculated control. Out of glucose and molasses, glucose was found to be better in terms of total VFA production.

As a second strategy, when the six Manikaran consortia were grown at 90°C from 80°C instead of directly going to 90°C from 70°C, on glucose (double strength), most of the consortia even after eleven cycles of enrichment showed erratic volatile fatty acid formation. During the later stages of enrichment, the VFA concentration stabilized around 1100 ppm. The consortia M-3, M-5 and M-6 showed better stabilization (Figure 24).

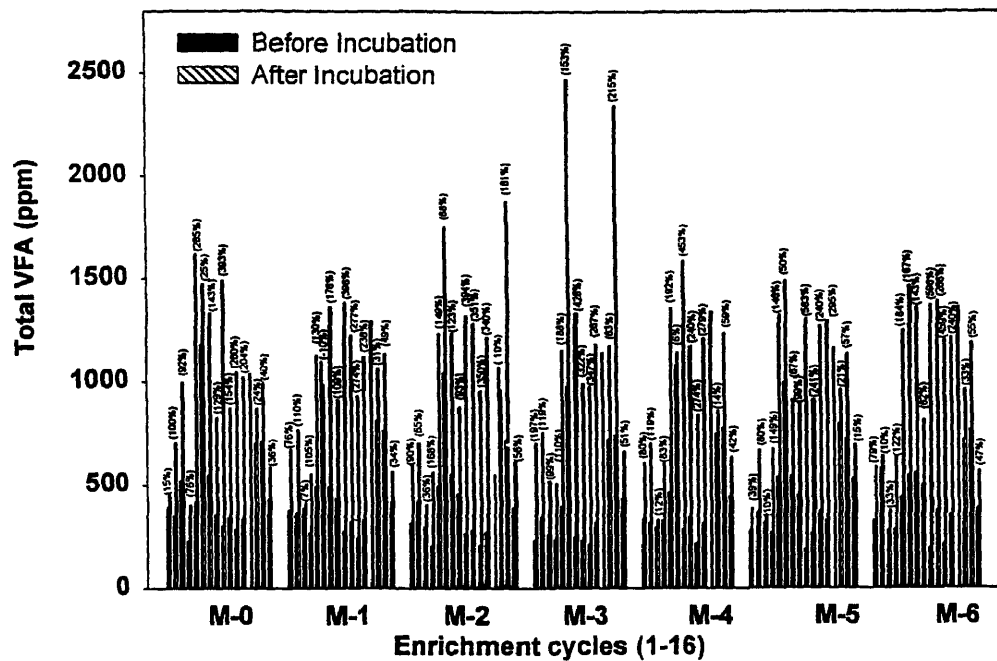


Figure 20. VFA production by Manikaran consortia at 90°C (70 to 90 °C) on glucose (double strength) as carbon source during various cycles of enrichment

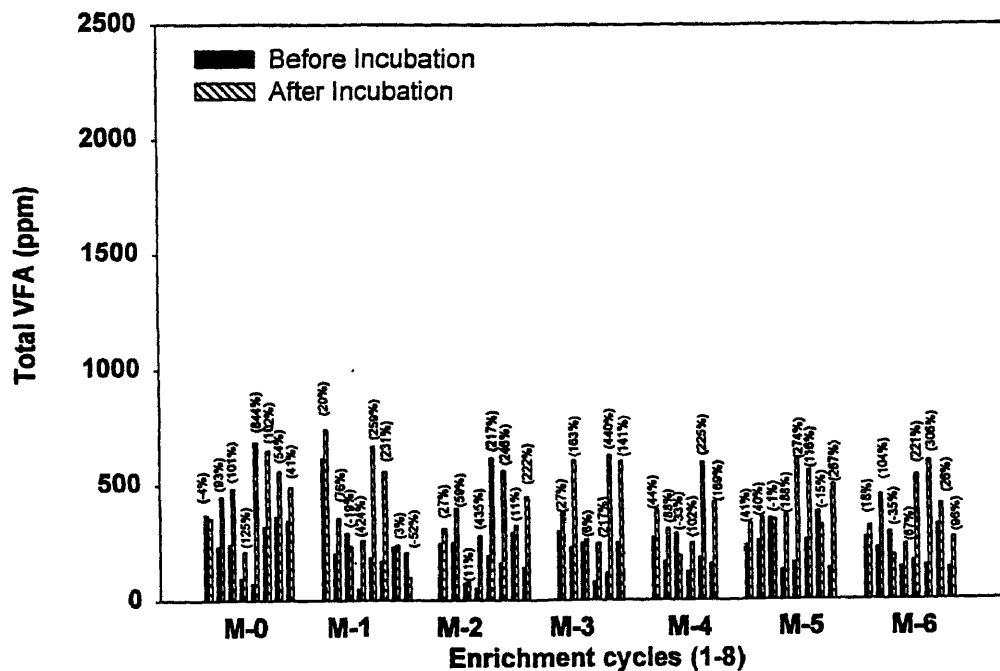


Figure 21. VFA production by Manikaran consortia at 90°C (70 to 90 °C) on molasses (double strength) as carbon source during various cycles of enrichment

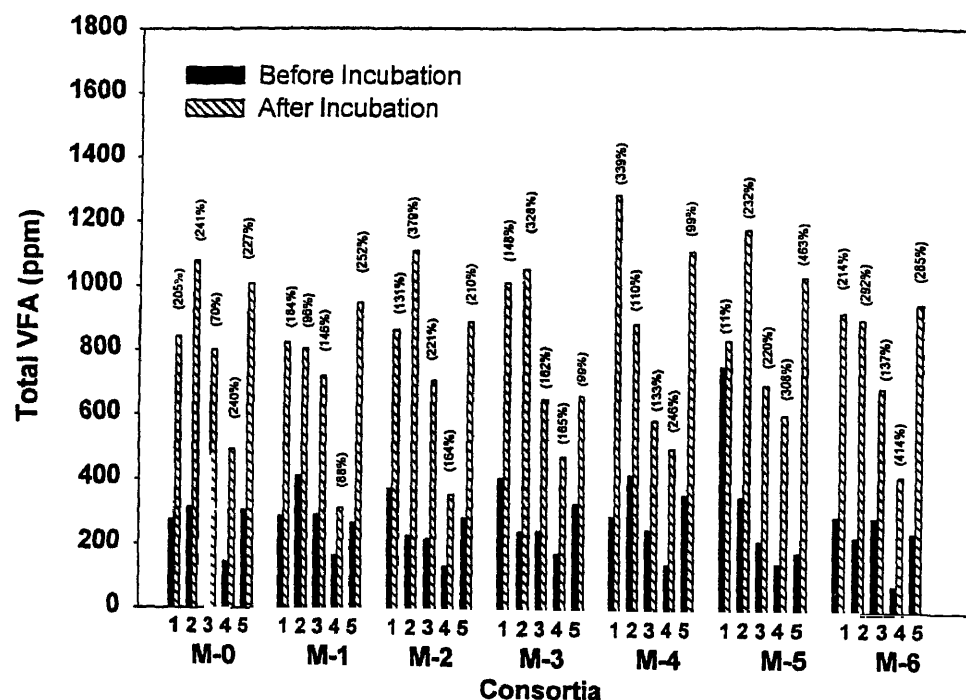


Figure 22. VFA production by Manikaran consortia at 85 °C on glucose (double strength) as carbon source during various cycles of enrichment

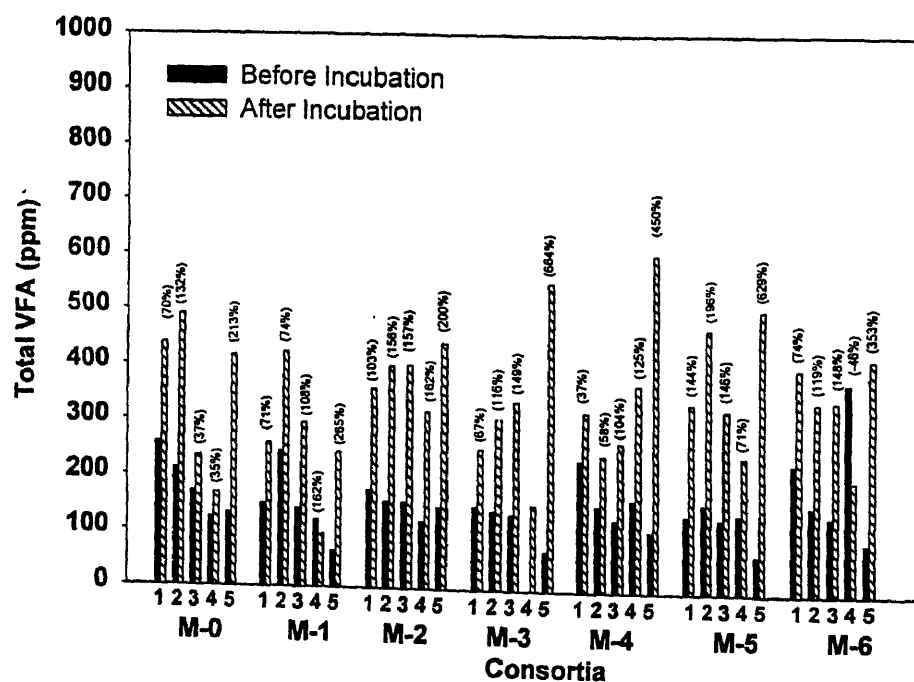


Figure 23. VFA production by Manikaran consortia at 85 °C on molasses (double strength) as carbon source during various cycles of enrichment

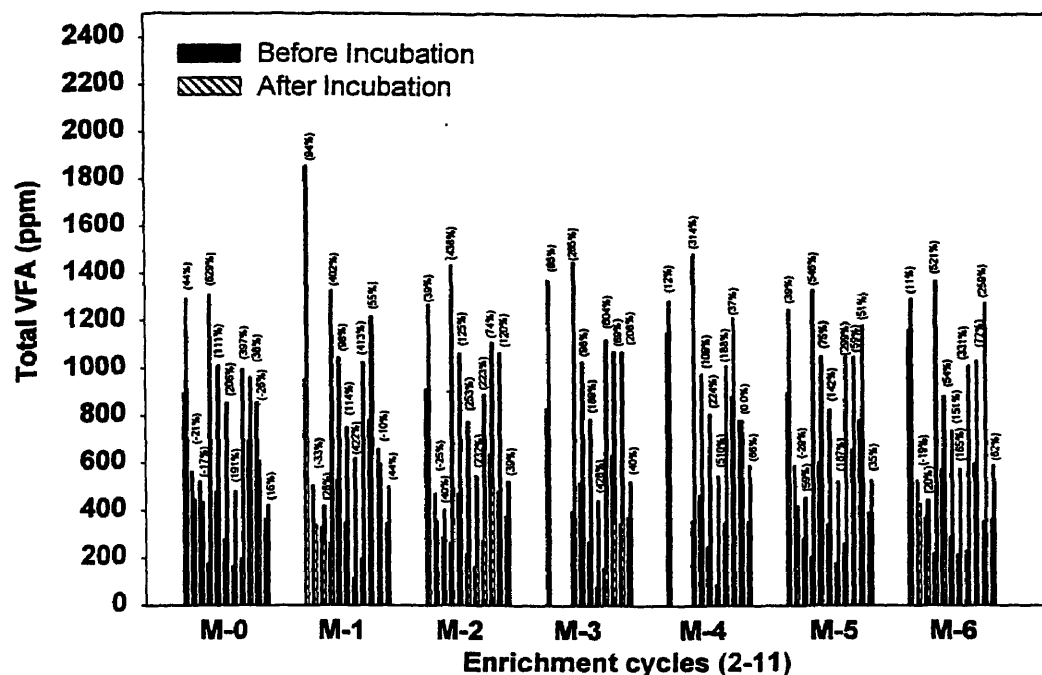


Figure 24. VFA production by Manikaran consortia at 90°C (80 to 90°C) on glucose (double strength) as carbon source during various cycles of enrichment

Four samples were collected from oil wells in Mehsana region of Gujarat. The characteristics of these samples are shown in Table 4. Among the four samples collected, two each from Sobhasan and Jotana, only consortia S-2 and J-1 were the best when grown on molasses single and double strength respectively on Balch *et al.* (1977) medium as compared to when grown on glucose at 70°C (Figures 25 to 28).

Table 4 Characteristics of the samples collected from Mehsana region of Gujarat

Sample No	Place	Source	Well temperature (°C)	Oil Content (%)	Colour
S-1	Sobhasan	Oil well	104	50	Blackish-brown
S-2	Sobhasan	Oil well	104	20	Blackish with pale yellow water
J-1	Jotana	Oil well No. 32	094	30	Yellowish- brown
J-2	Jotana	Oil well No. 109	094	95	Brownish -black

Analysis of volatile fatty acid production when the consortia were grown at 80°C with glucose and molasses indicated that among the two strengths with both the

single strength for both the consortia, S-2 and J-1 (Figures 29 to 31). Further among the two carbon sources at double strength glucose was better than molasses with respect to the amounts of total volatile fatty acids. Therefore glucose as a carbon source was selected for further studies at both 80 and 90°C

These consortia were then enriched at 90°C and enhanced production of volatile fatty acids (over zero time) was observed over the un-inoculated control therefore indicating that the microbial consortia were gradually enriched at 90°C (Figure 32). During the later enrichment cycles, an increase of 50 to 75% in total volatile fatty acids was observed.

Further enrichment over another ten cycles confirmed that the consortium S-2 and J-1 showed 300 to 500 ppm increased VFA production over zero time. Another consortium J-2 also was being enriched at 90°C, when grown on glucose showed acetic acid as the only volatile fatty acid produced indicating gradual enrichment of thermophilic consortia. Consortium J-1 and S-2 were finally selected for initial sand pack column studies.

The various consortia from Mehsana, when grown at 85°C on glucose (double strength) indicated that S-2 was better both in terms of % increase in VFA production in comparison to changes observed in un-inoculated control (A-0) due to high temperature and also in terms of stability (Figure 33).

When the same consortia were grown on molasses (single strength), the total VFA production was found to be 50-75% less (Figure 34) in comparison to the respective consortium with glucose (double strength) as carbon source. Although, initially both S-2 and J-1 showed less VFA formation in comparison to J-2 but both indicated improved VFA formation in the subsequent cycles.

When the same consortia from Mehsana were transferred from 80°C to 90°C, during various cycles of enrichment at 90°C, J-2 invariably showed higher % increase in VFA over 0 time and with un-inoculated control (A-0) with glucose (double strength) as carbon source (Figure 35).

Again out of the two carbon sources viz., glucose (double strength) and molasses (single strength), glucose (double strength) was found to be better in terms of total VFA production.

With molasses, the VFA production was found to be more erratic (Figure 36) in comparison to glucose.

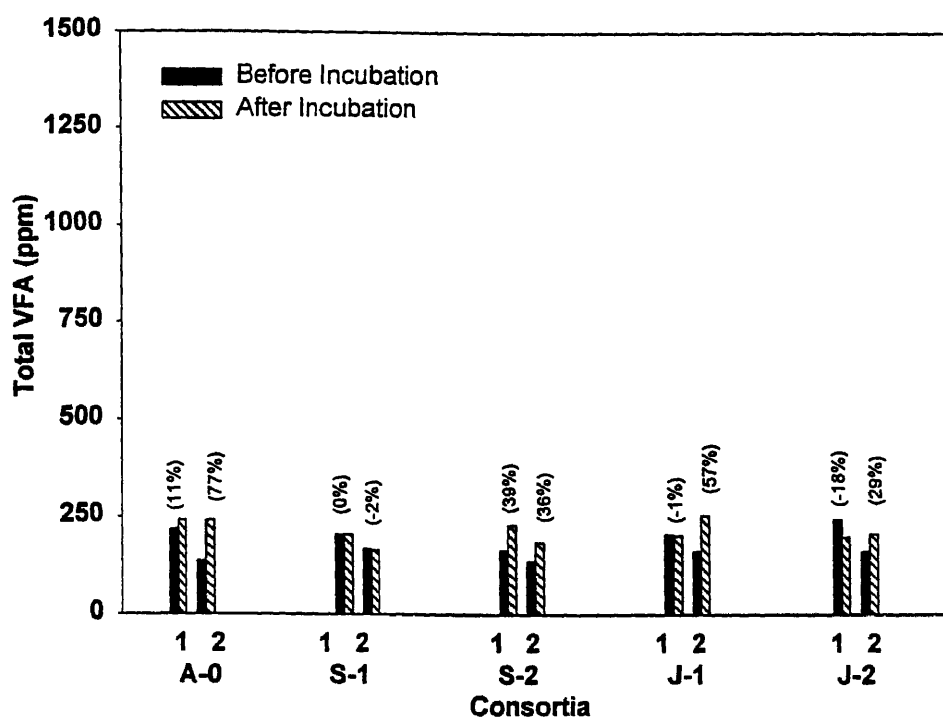


Figure 25. VFA production by Mehsana consortia at 70 °C on glucose (single strength) as carbon source during various cycles of enrichment

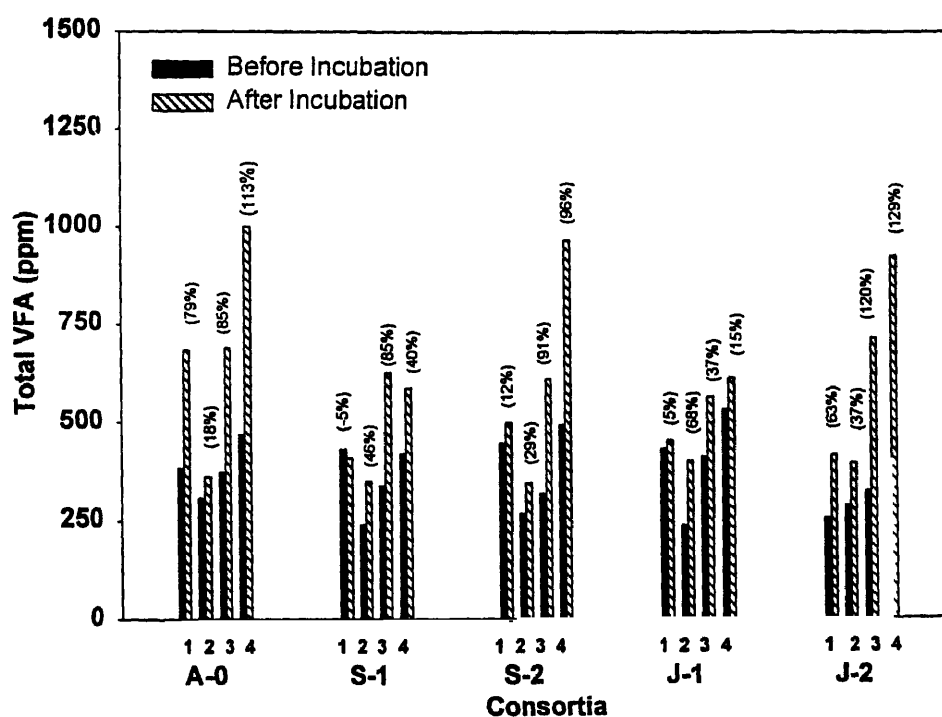


Figure 26. VFA production by Mehsana consortia at 70 °C on glucose (double strength) as carbon source during various cycles of enrichment

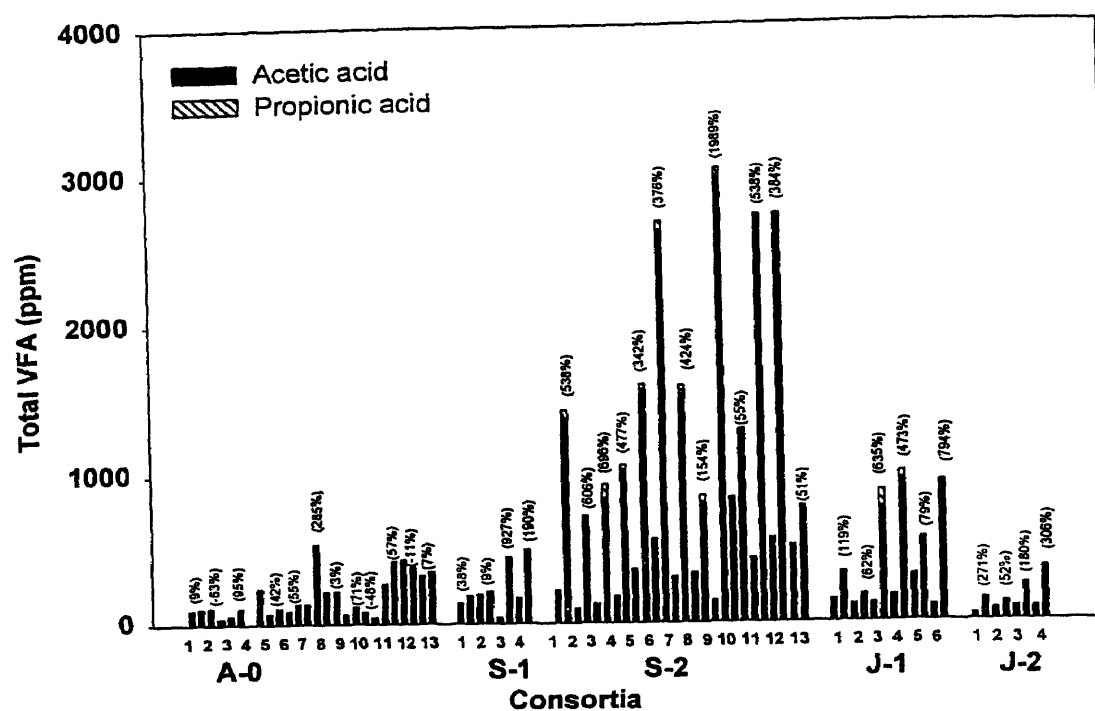


Figure 27. VFA production by Mehsana consortia at 70 °C on molasses (single strength) as carbon source during various cycles of enrichment

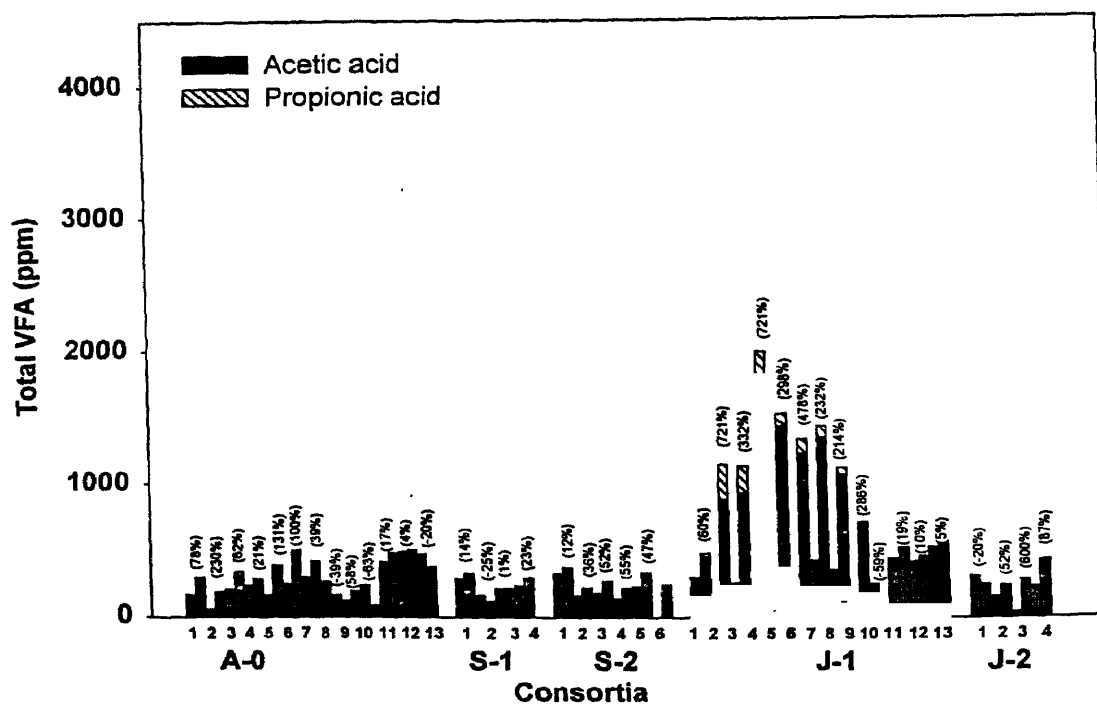


Figure 28. VFA production by Mehsana consortia at 70 °C on molasses (double strength) as carbon source during various cycles of enrichment

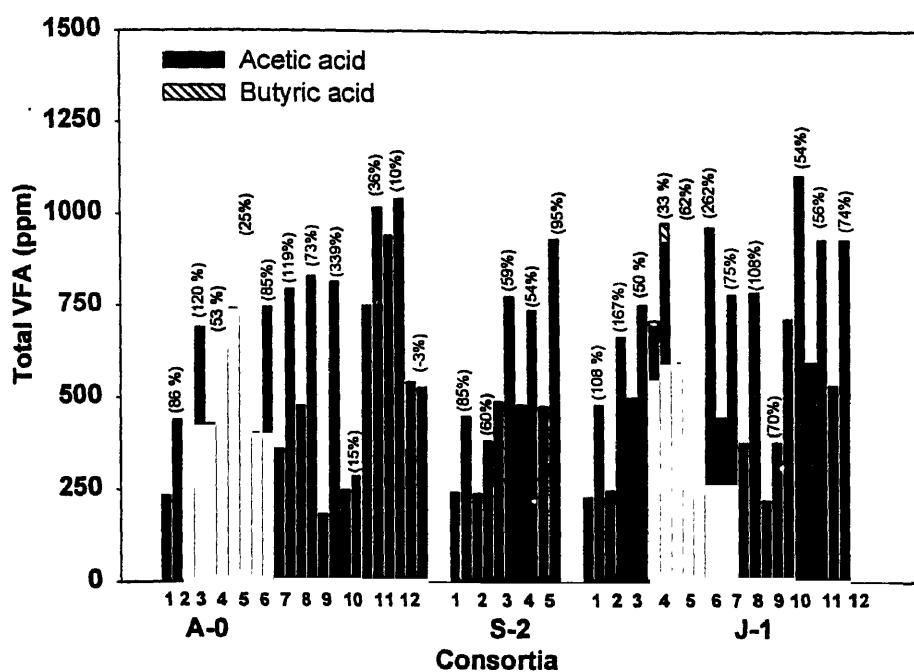


Figure 29. VFA production by Mehsana consortia at 80°C on glucose (double strength) as carbon source during various cycles of enrichment

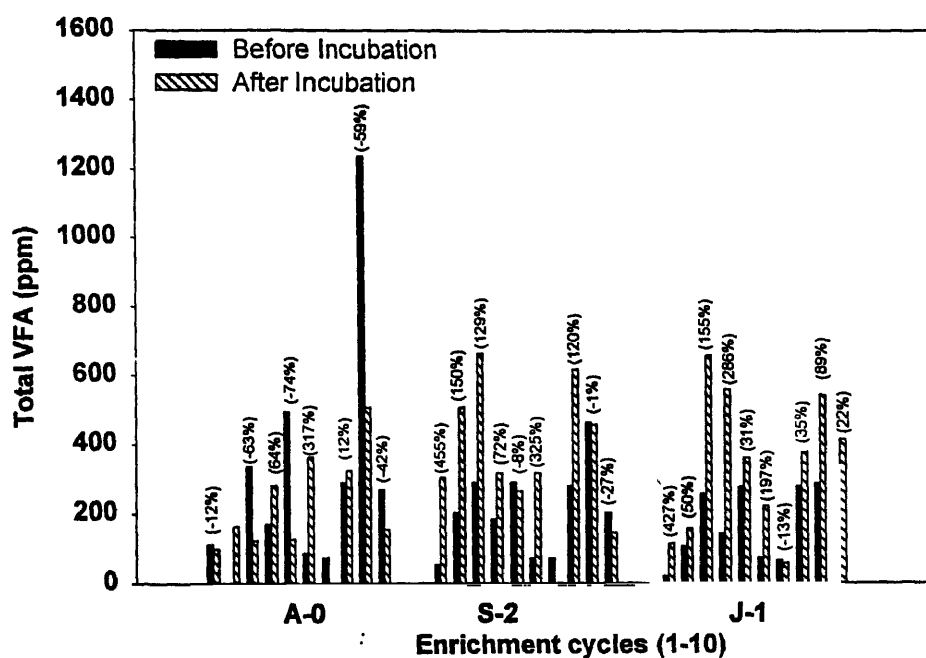


Figure 30. VFA production by Mehsana consortia at 80°C on molasses (single strength) as carbon source during various cycles of enrichment

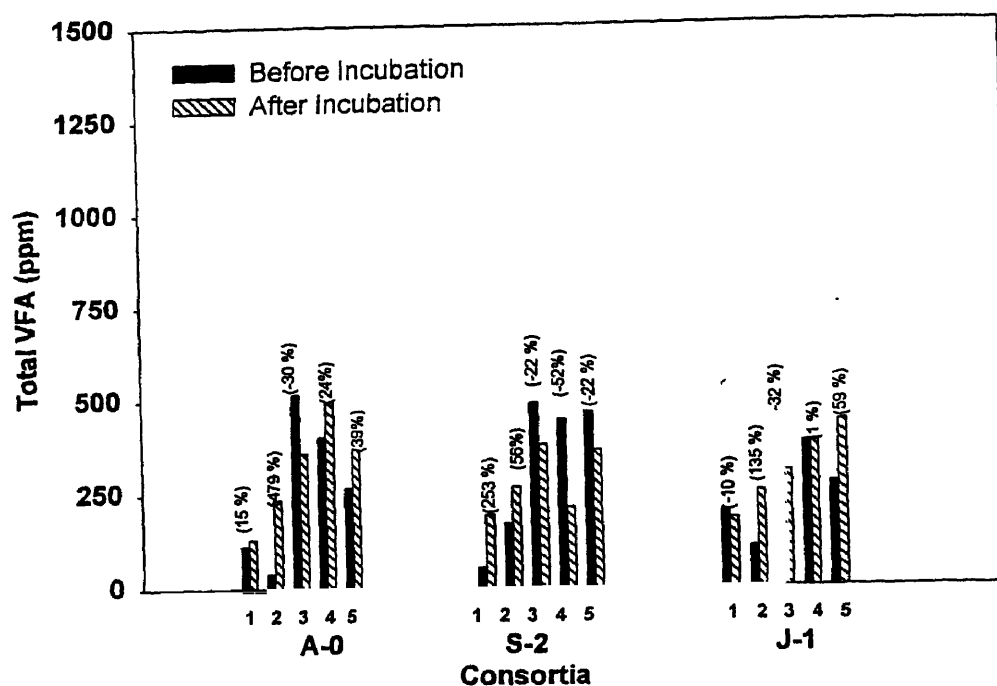


Figure 31. VFA production by Mehsana consortia at 80°C on molasses (double strength) as carbon source during various cycles of enrichment

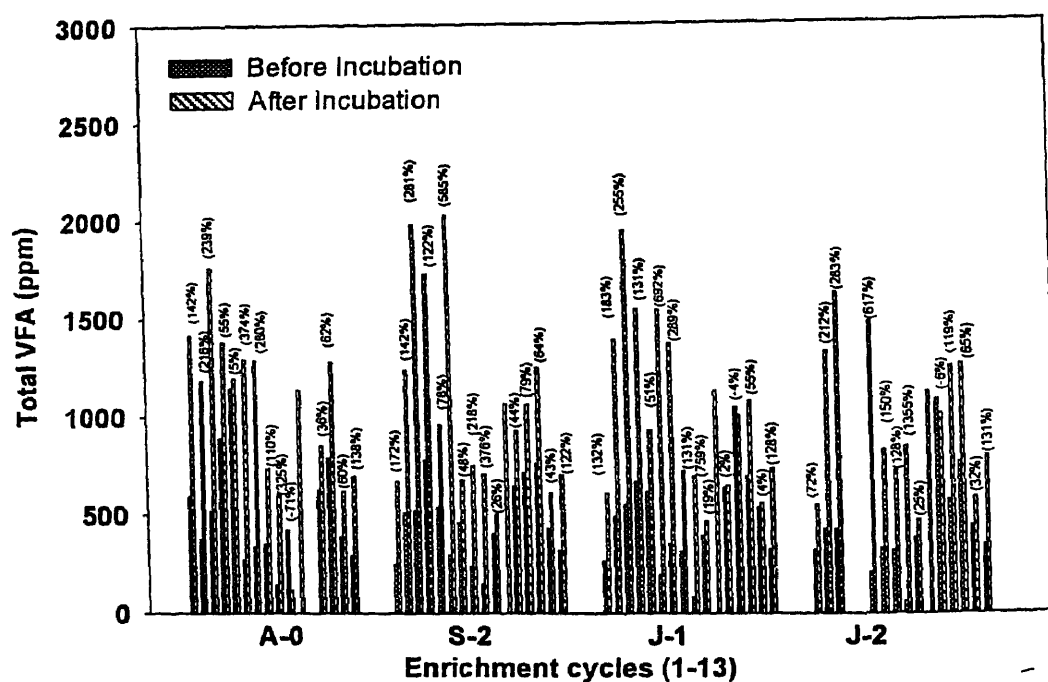


Figure 32. VFA production by Mehsana consortia at 90°C on glucose (double strength) as carbon source during various cycles of enrichment

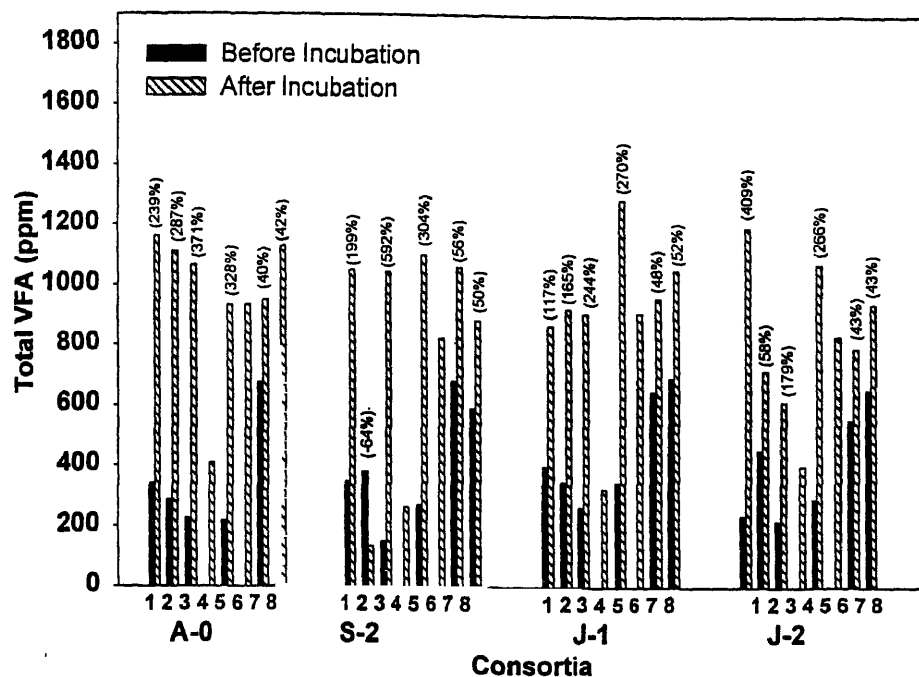


Figure 33. VFA production by Mehsana consortia at 85 °C on glucose (double strength) as carbon source during various cycles of enrichment

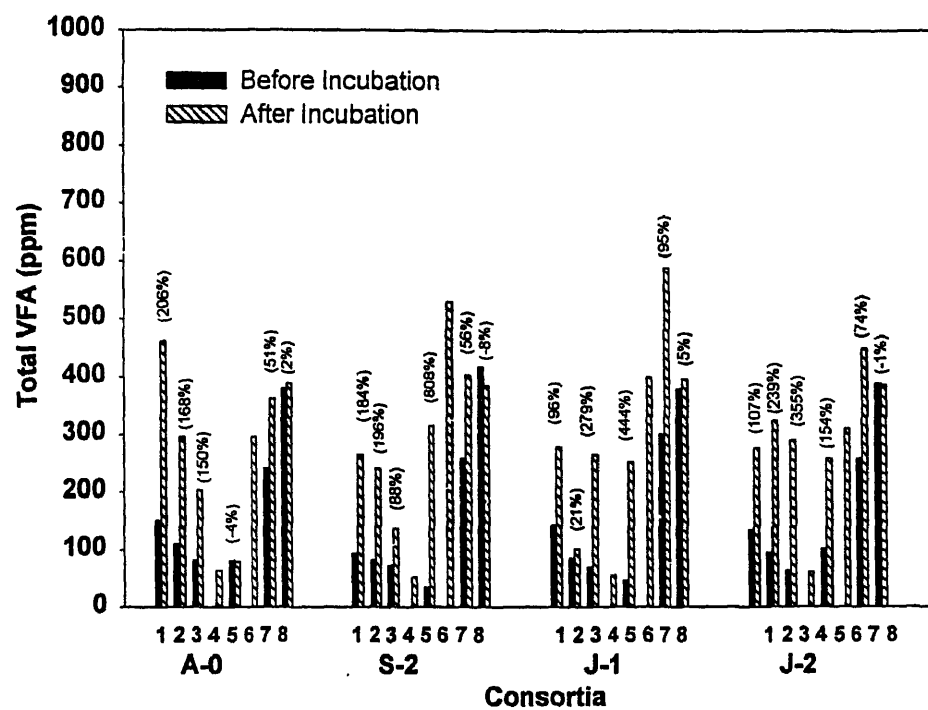


Figure 34. VFA production by Mehsana consortia at 85 °C on molasses (single strength) as carbon source during various cycles of enrichment

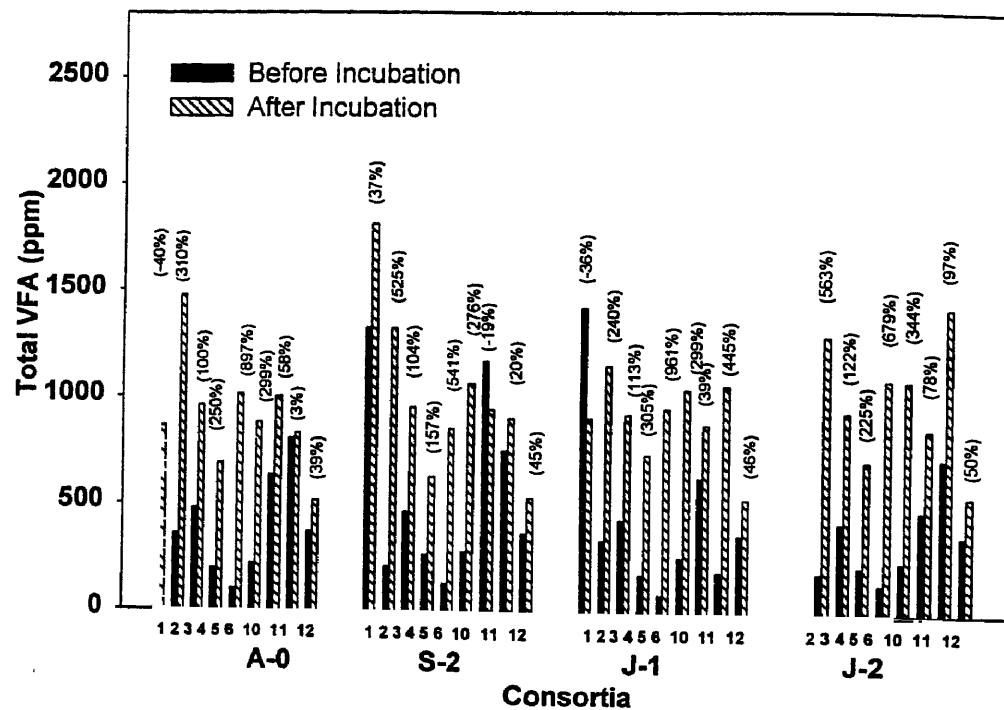


Figure 35. VFA production by Mehsana consortia at 90°C (80 to 90 °C) on glucose (double strength) as carbon source during various cycles of enrichment

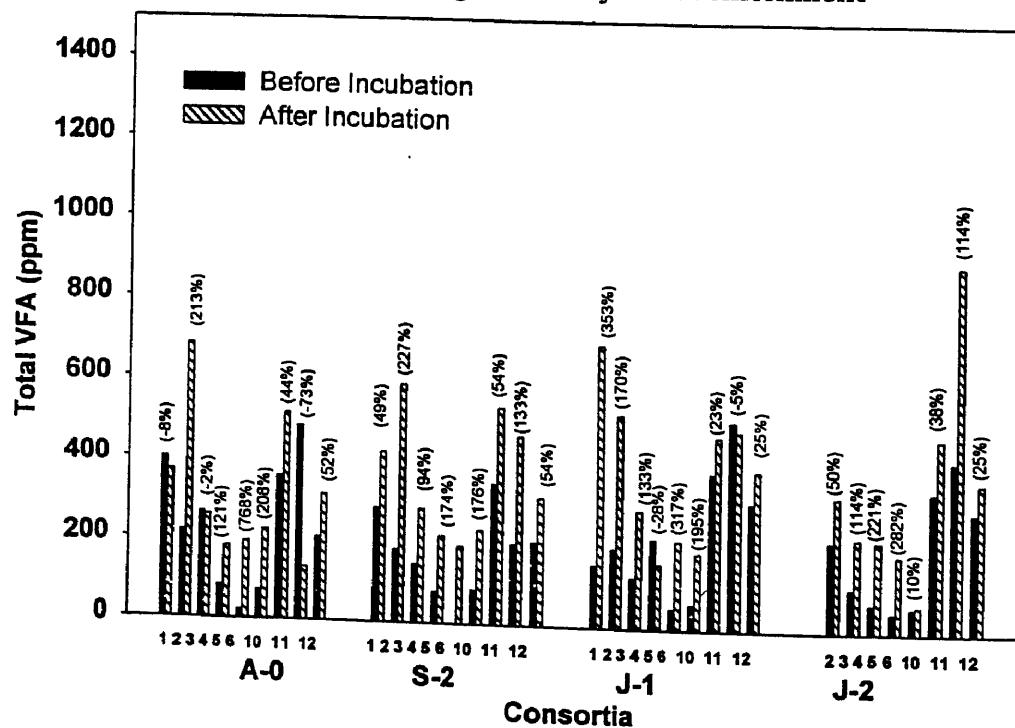


Figure 36. VFA production by Mehsana consortia at 90°C (80 to 90 °C) on molasses (single strength) as carbon source during various cycles of enrichment

The characteristics of the various samples collected from the Konkan region, Maharashtra are shown in Table 5. Two consortia, Uw and Rj were the best among the six consortia tested at 70°C for development of anaerobic, thermophilic consortia both on glucose and molasses as carbon sources in single and double strength using Balch *et al.* (1977) medium for volatile fatty acid production (Figures 37 to 40). The consortium Uw showed an increase of over 500%, producing around 5000 ppm volatile fatty acid when grown on molasses (double strength) while when grown on glucose the total volatile fatty acid produced were only between 2000 to 3000 ppm. Among the two carbon sources tested for growth, molasses both in single and double strength showed higher volatile fatty acid production, almost twice to that observed in glucose (both single and double strength). Thus, molasses was found to be superior for both growth and total volatile fatty acid production in comparison to glucose. Therefore, further enrichment at 70°C was continued only with molasses (single and double strengths). A comparison of the various consortia developed at 70°C from Manikaran, Mehsana and Konkan region indicated two unique features in the Uw consortia from Konkan region. One that the amount of volatile fatty acids produced by the Uw consortia was much higher as compared to the consortia from Manikaran and Mehsana regions. Two that the profile of the volatile fatty acids produced by consortia Uw from Konkan region at 70°C also showed the presence of iso-butyric and butyric acids apart from acetic and propionic acids. Acetic and occasionally propionic acids were the only acids being produced by consortia from Manikaran and Mehsana regions. The enriched consortia at 70°C from the Konkan region were further enriched and adapted at 80, 85, and 90°C.

Table 5 Characteristics of the samples collected from Konkan region of Maharashtra

Sample		Sample		
No	Place	Source	Temperature (°C)	Sample texture and Colour
G	Ganeshpun	Hot water spring	50-55	Clear water with grey black sand crystals along with yellow colour rice
V-1	Vajreshwari	Hot water spring	45-47	Clear water with black sand crystals
V-2	Vajreshwari	Hot water spring	45-47	Clear water with less quantity of black sand crystals
Un	Unhere	Hot water spring	42-45	Clear water with dark black sand crystals and pallets
Uw	Unwahere	Hot water spring	70-80	Highly turbid water with brown-black soil
Rj-1	Rajewadi	Hot water spring	62-70	Clear -slightly turbid water with greyish-black soil
Rj-2	Rajewadi	Hot water spring	62-65	Highly turbid water with light brown soil and rich in dark brown plant residues.

None of the consortium were very active at 80°C except Uw which consistently showed good volatile fatty acid production when grown on molasses although the amount of volatile fatty acid produced were lower (Figures 41 to 44) than that at 70°C.

At 90°C consortium V-2 showed higher volatile fatty acid production when grown on molasses. This consortium was further enriched at 90°C (Figures 45 to 48).

All the consortia collected from Konkan region were transferred from 80°C to 85°C on both glucose and molasses (single and double strength). During the enrichment at 85°C, out of the two carbon sources, in terms of total VFA production, glucose (double strength) was found to be better than molasses and all other concentrations. During the three initial cycles of enrichment, the VFA formation was found to be erratic, invariably by all the consortia with both the carbon sources (Figures 49 to 52).

Both glucose and molasses (double strength) were found to give higher VFA formation at 90°C (Figures 53 to 56) compared to the respective consortia with single strength carbon sources, when all the consortia collected from Konkan region were transferred from 80°C to 90°C on both glucose and molasses (single and double strength). However among the various consortia no definite trend could be observed.

About 50% reduction in VFA production was observed with molasses (double strength) in comparison to the respective consortia on glucose (double strength).

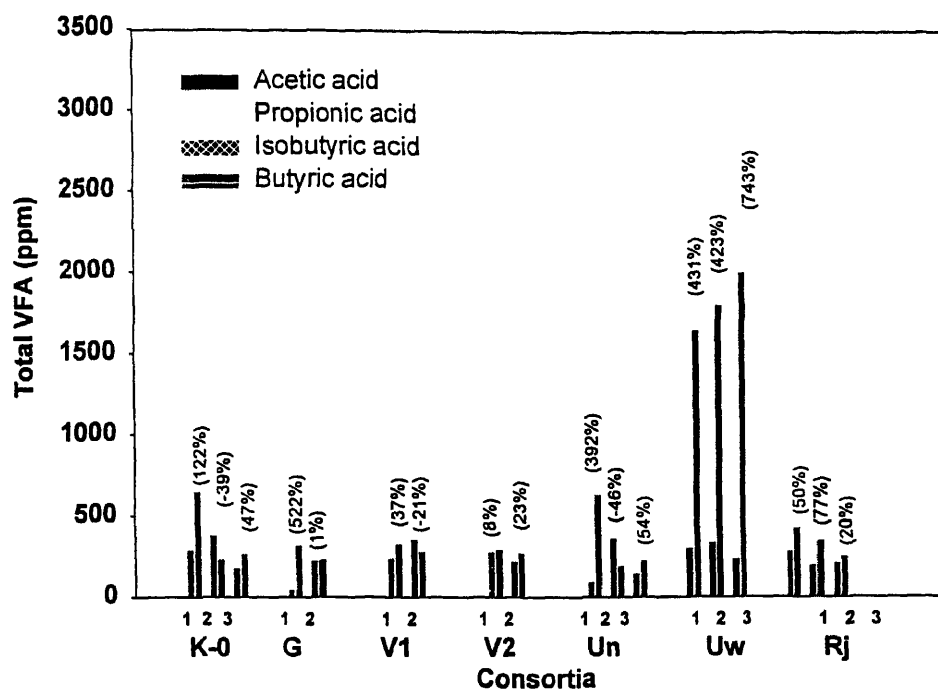


Figure 37. VFA production by Konkan consortia at 70°C on glucose (single strength) as carbon source during various cycles of enrichment

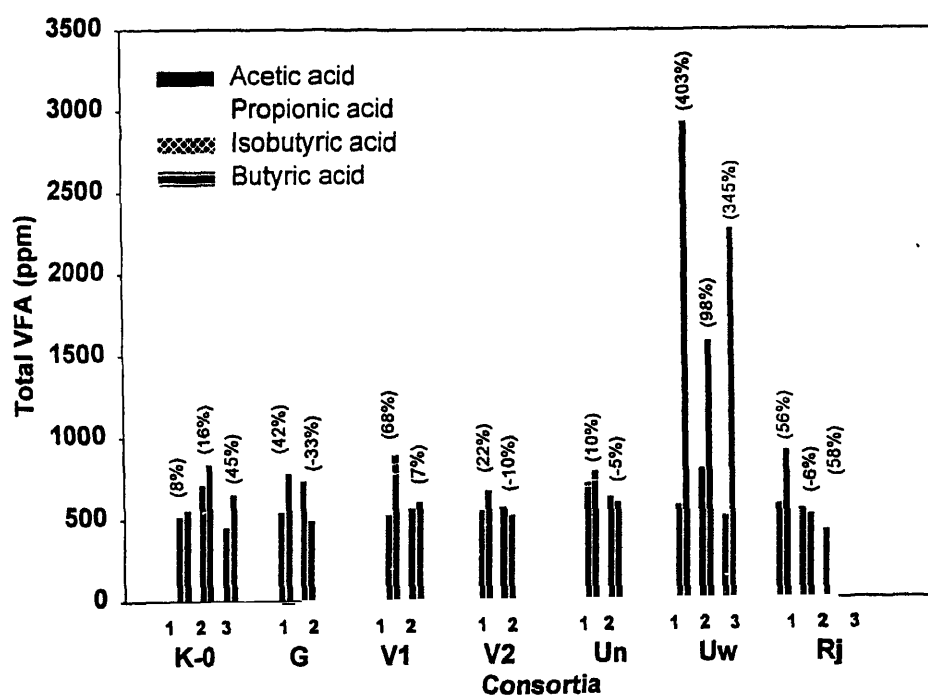


Figure 38. VFA production by Konkan consortia at 70°C on glucose (double strength) as carbon source during various cycles of enrichment

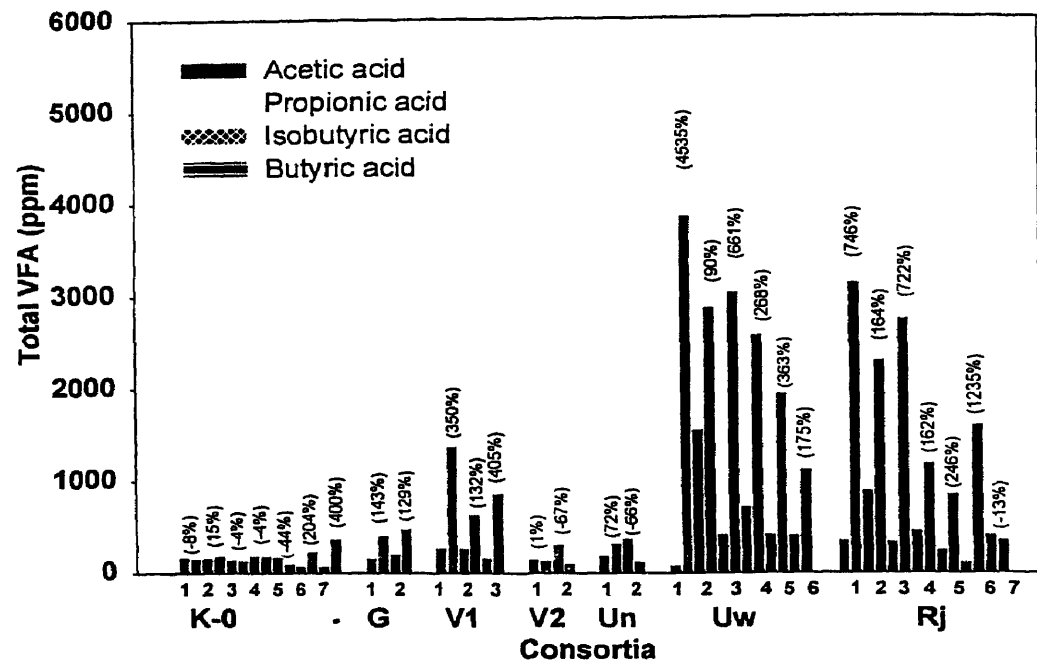


Figure 39. VFA production by Konkani consortia at 70 °C on molasses (single strength) as carbon source during various cycles of enrichment

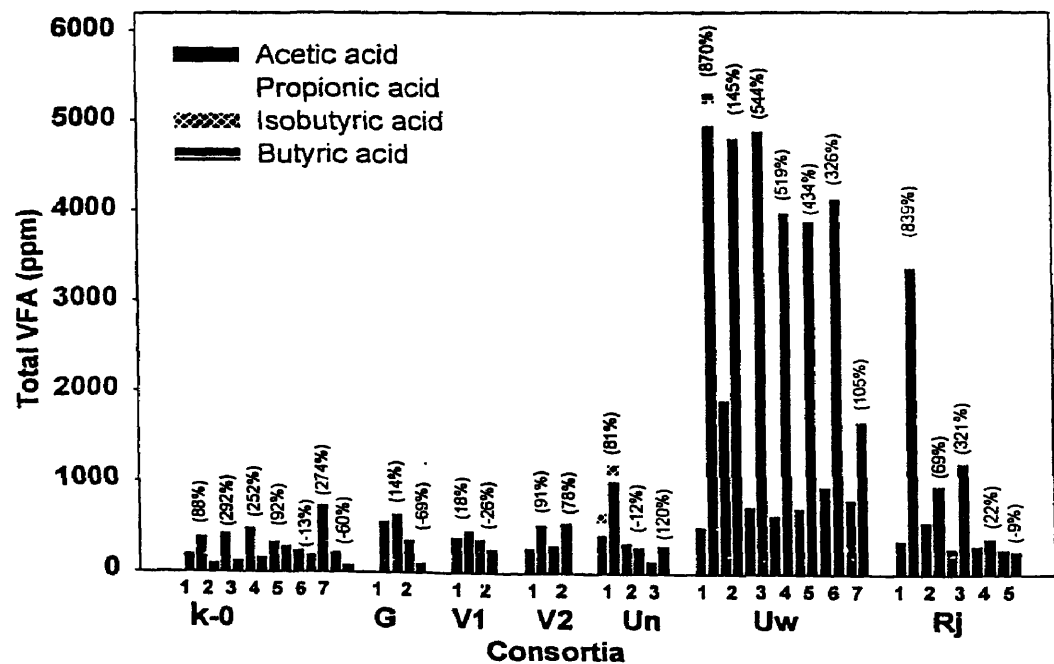


Figure 40. VFA production by Konkani consortia at 70 °C on molasses (double strength) as carbon source during various cycles of enrichment

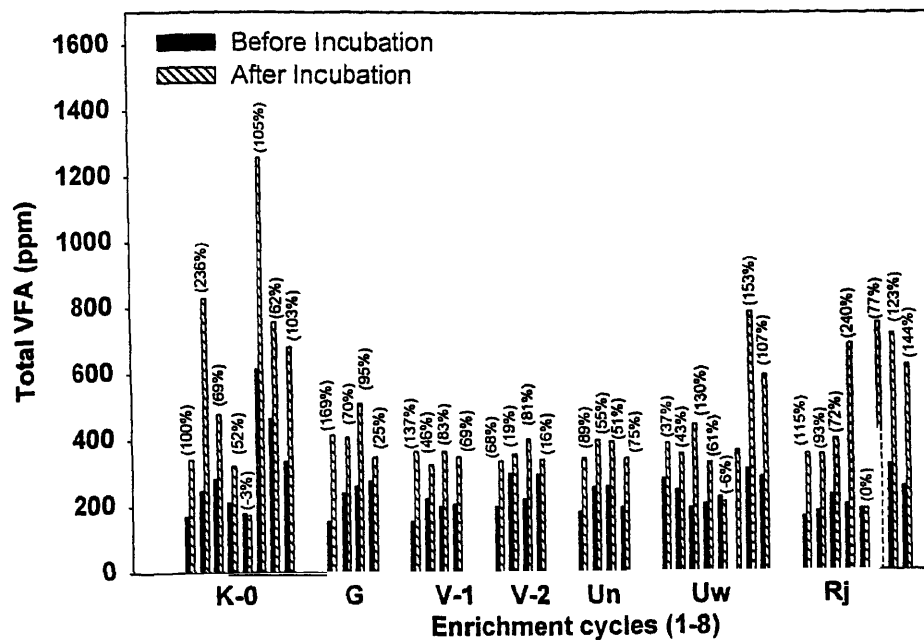


Figure 41. VFA production by Konkan at 80 °C on glucose (single strength) as carbon source during various cycles of enrichment

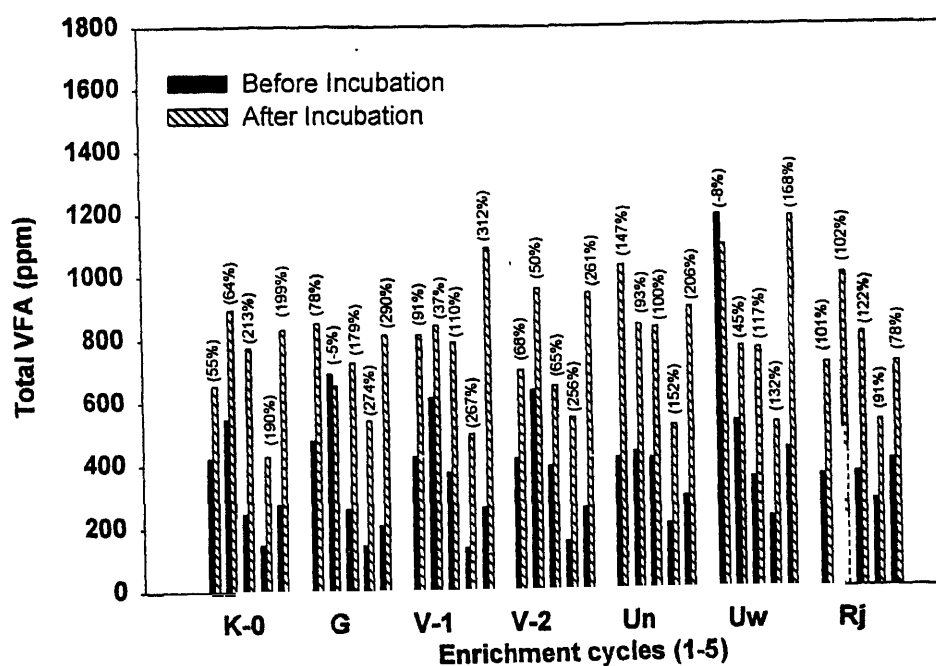


Figure 42. VFA production by Konkan at 80 °C on glucose (double strength) as carbon source during various cycles of enrichment

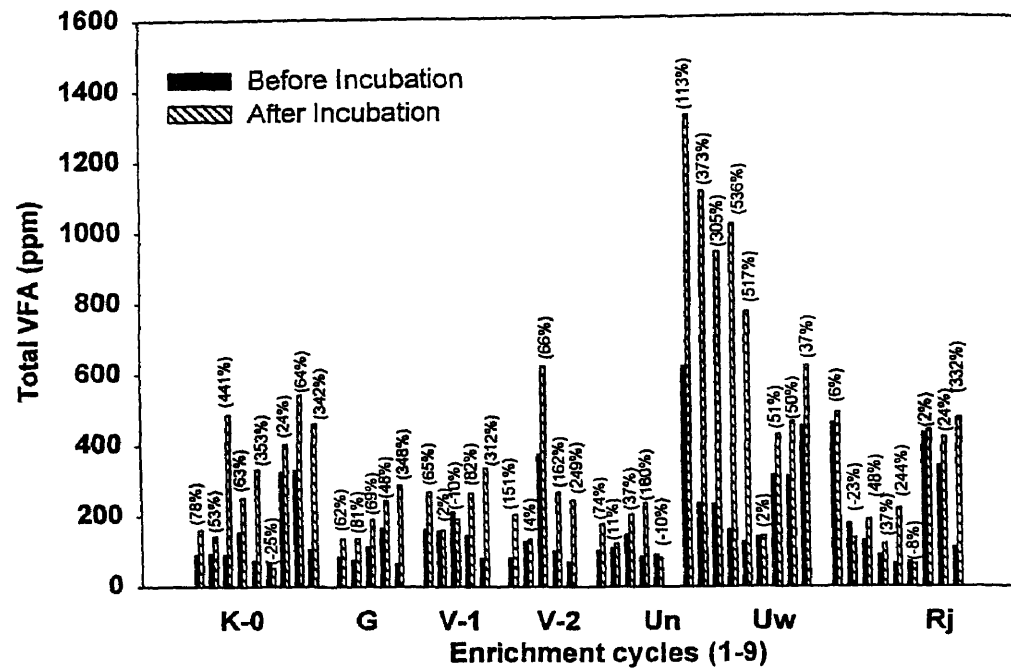


Figure 43. VFA production by Konkani consortia at 80°C on molasses (single strength) as carbon source during various cycles of enrichment

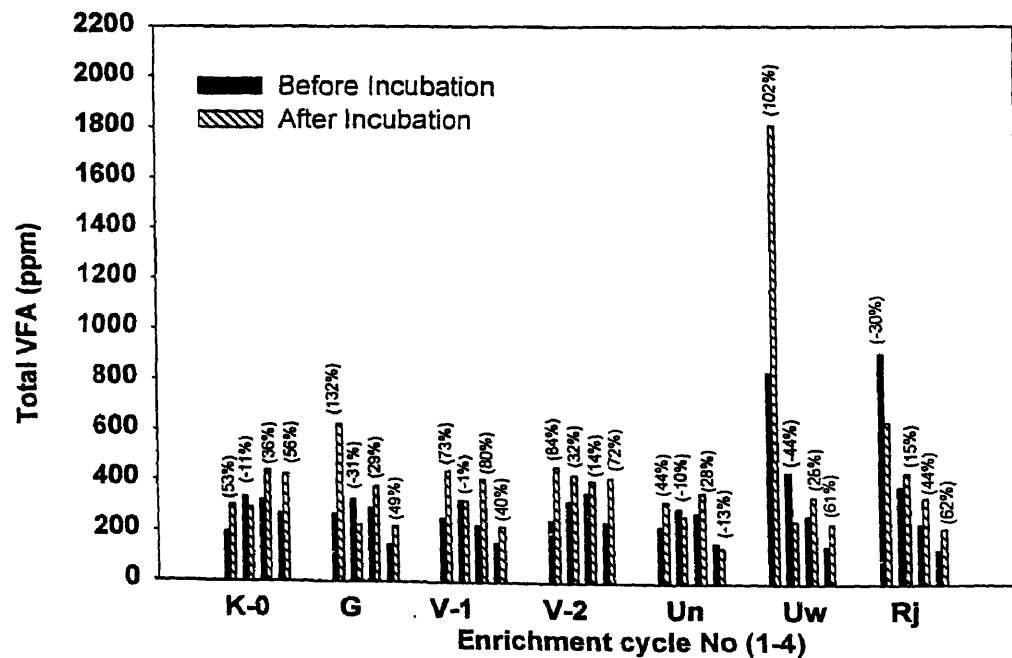


Figure 44. VFA production by Konkani consortia at 80°C on molasses (double strength) as carbon source during various cycles of enrichment

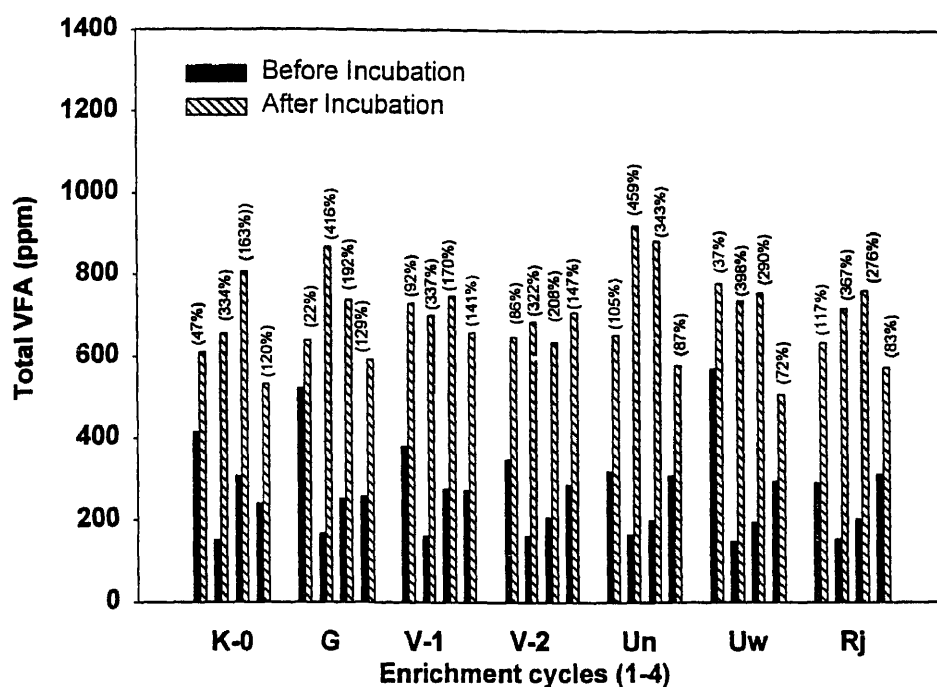


Figure 45. VFA production by Konkan consortia at 90°C on glucose (single strength) as carbon source during various cycles of enrichment

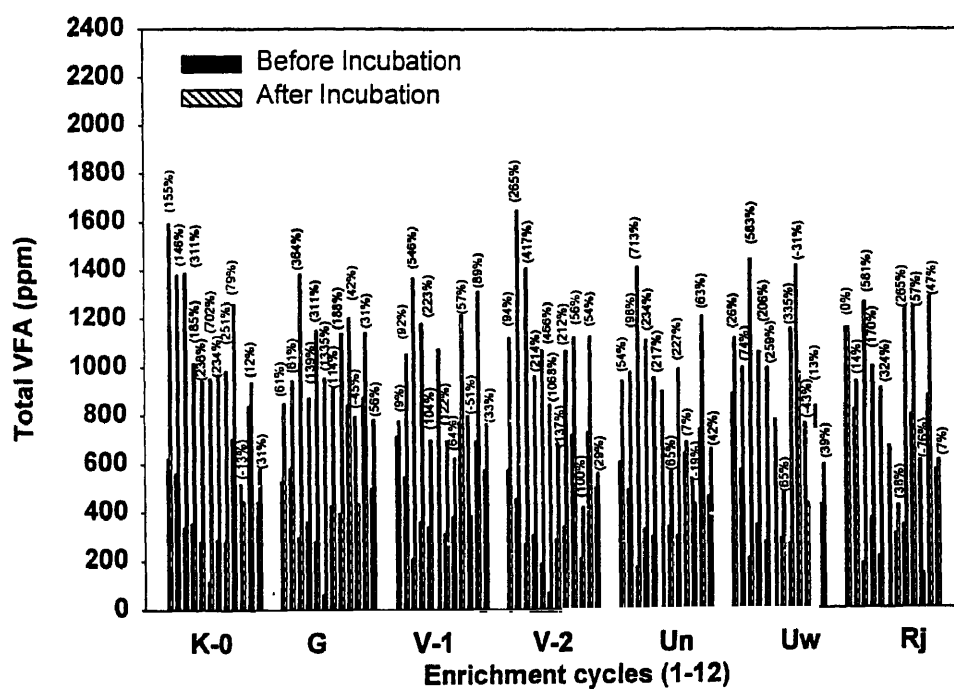


Figure 46. VFA production by Konkan consortia at 90°C on glucose (double strength) as carbon source during various cycles of enrichment

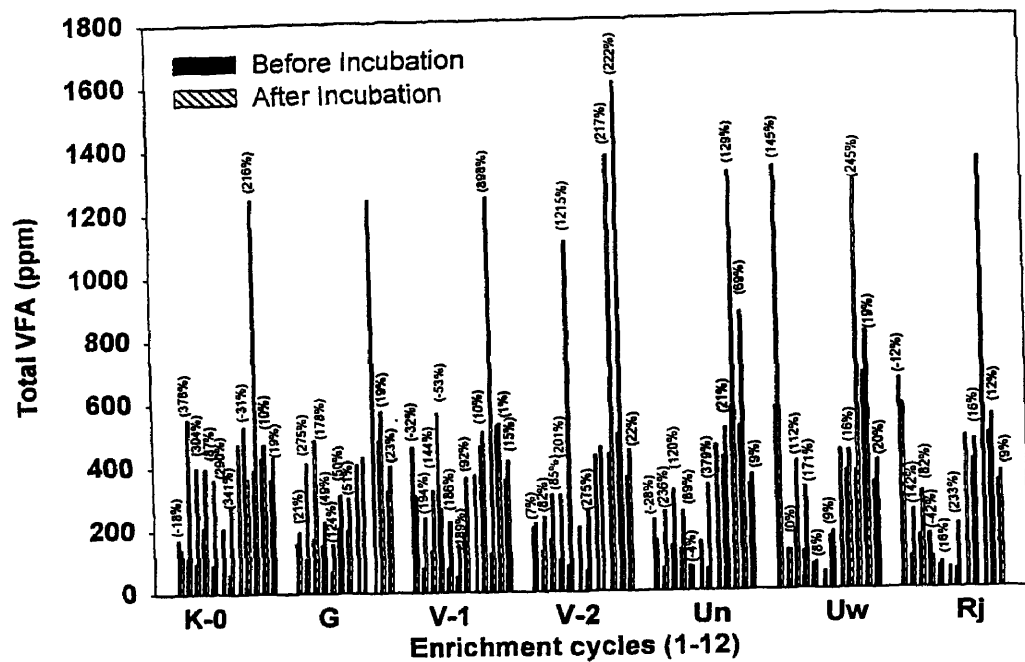


Figure 47. VFA production by Konkani consortia at 90 °C on molasses (single strength) as carbon source during various cycles of enrichment

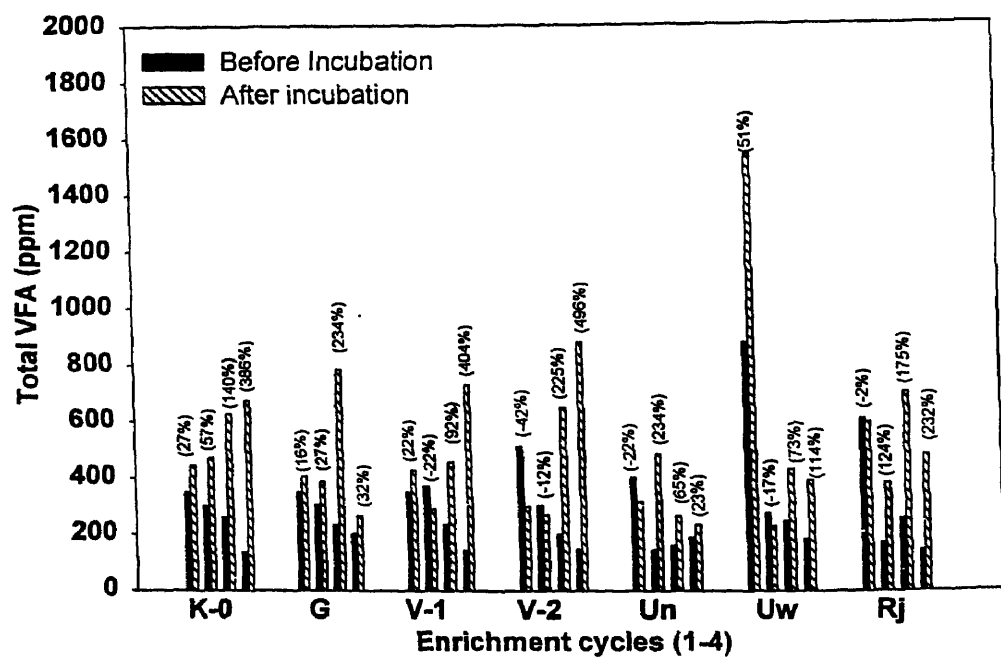


Figure 48. VFA production by Konkani consortia at 90 °C on molasses (double strength) as carbon source during various cycles of enrichment

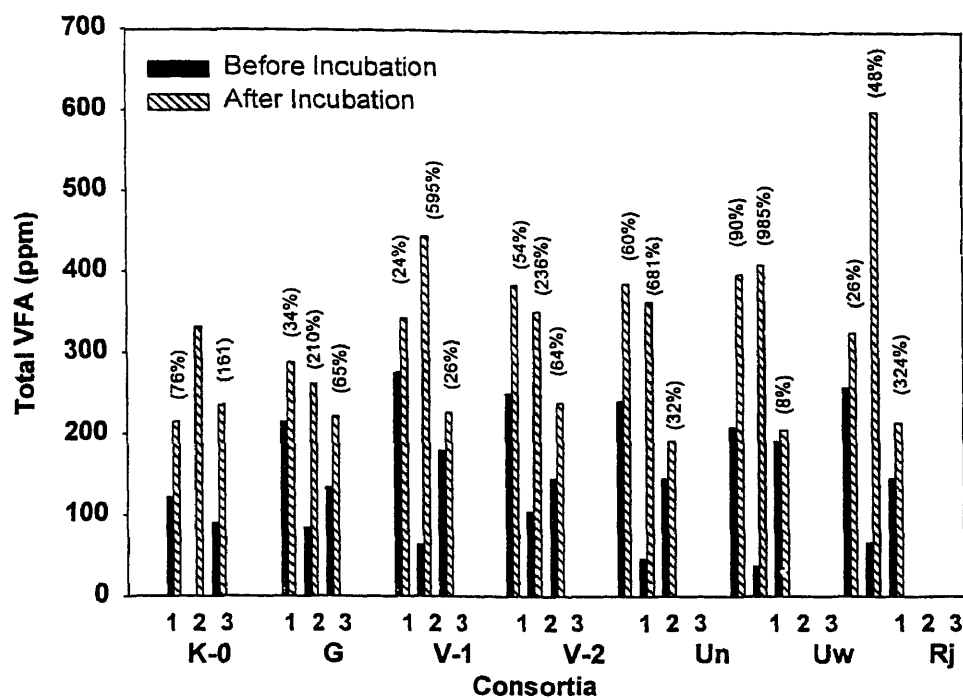


Figure 49. VFA production by Konkani consortia at 85 °C on glucose (single strength) as carbon source during various cycles of enrichment

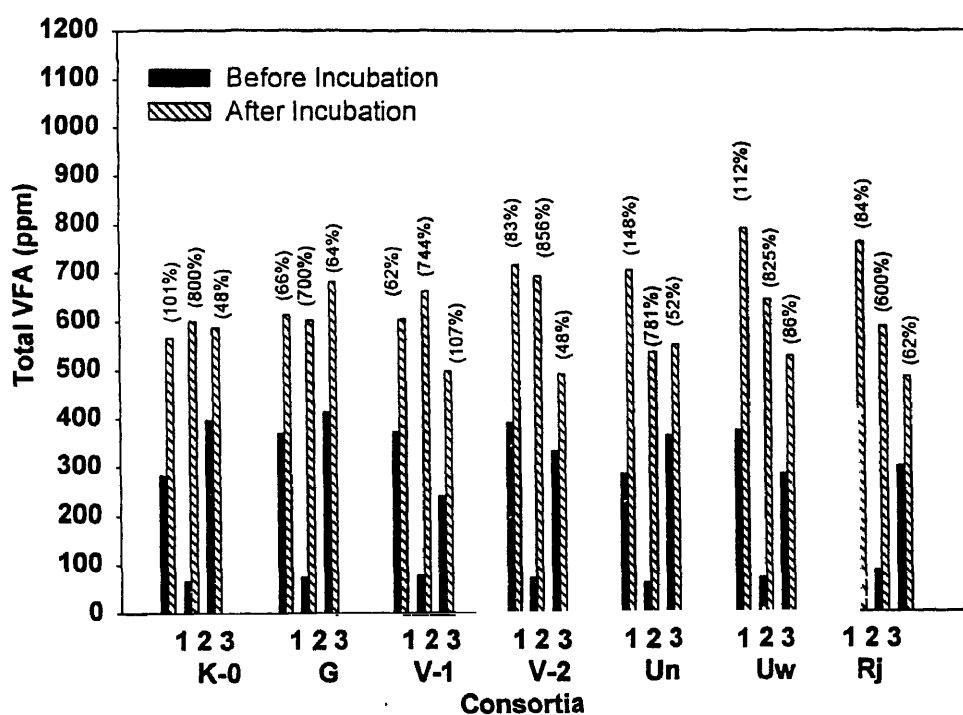


Figure 50. VFA production by Konkani consortia at 85 °C on glucose (double strength) as carbon source during various cycles of enrichment

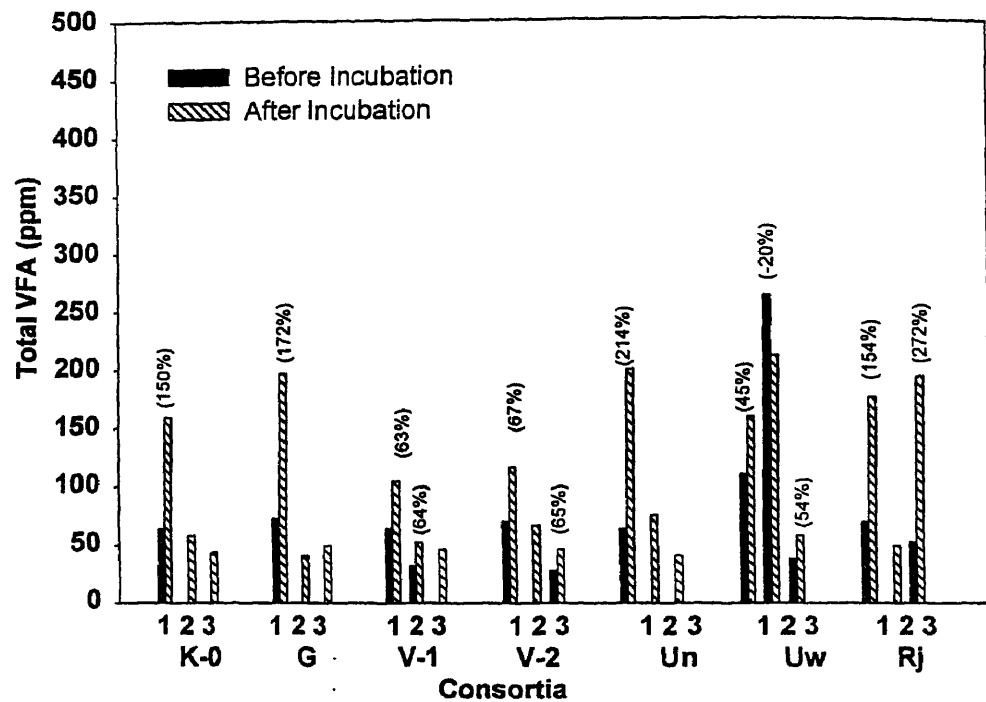


Figure 51. VFA production by Konkani consortia at 85 °C on molasses (single strength) as carbon source during various cycles of enrichment

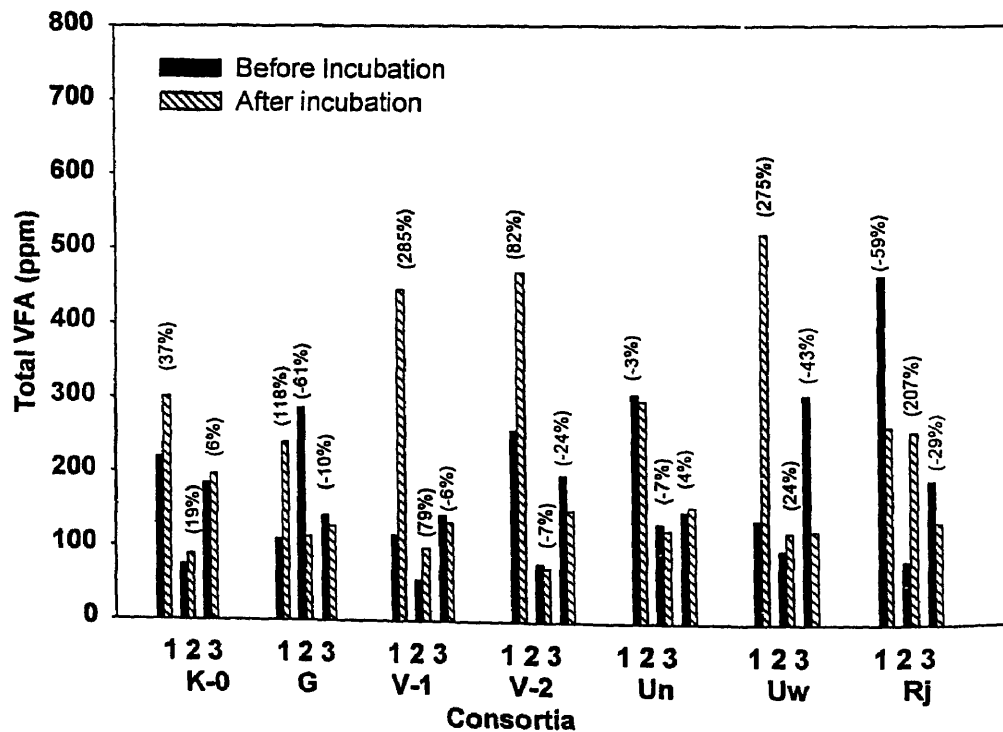


Figure 52. VFA production by Konkani consortia at 85 °C on molasses (double strength) as carbon source during various cycles of enrichment

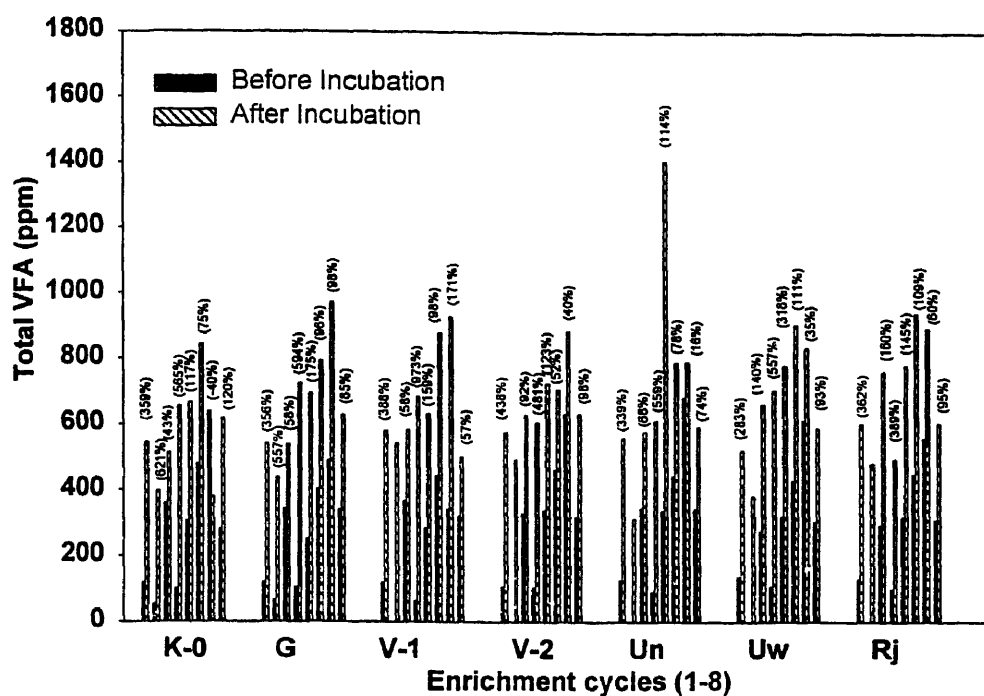


Figure 53. VFA production by Konkani consortia at 90°C (80 to 90 °C) on glucose (single strength) as carbon source during various cycles of enrichment

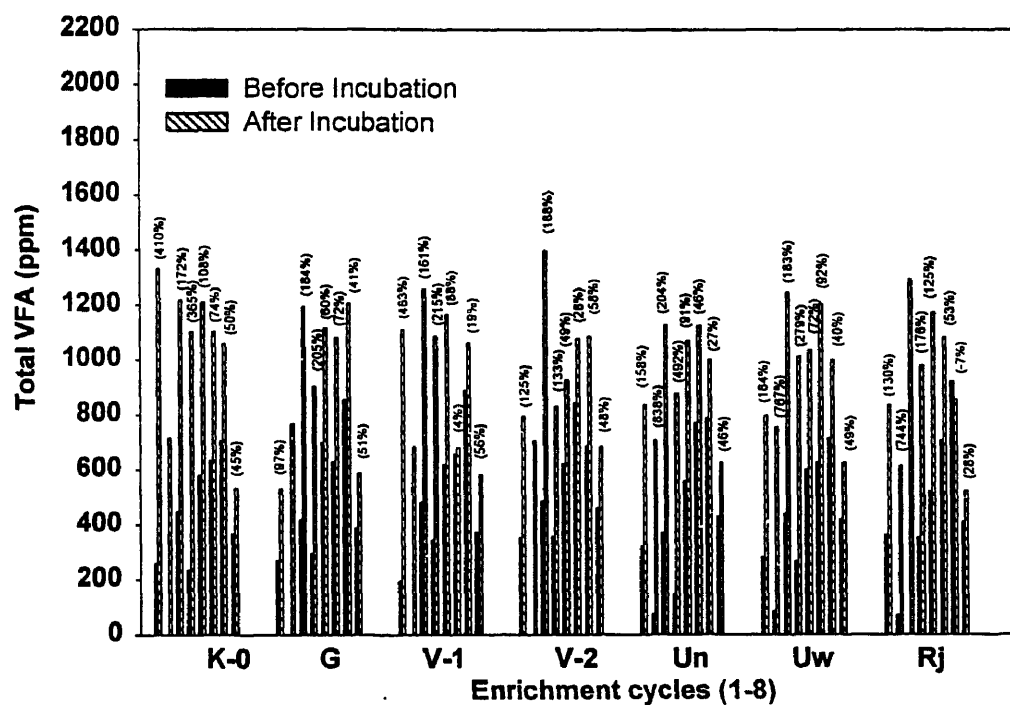


Figure 54. VFA production by Konkani consortia at 90°C (80 to 90 °C) on glucose (double strength) as carbon source during various cycles of enrichment

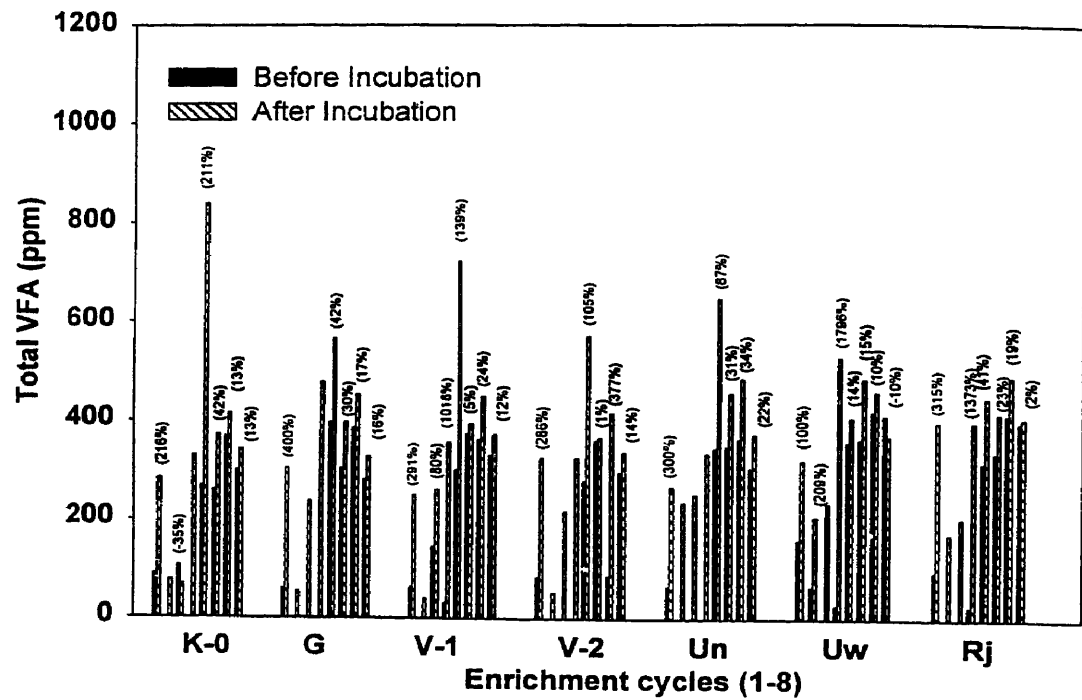


Figure 55. VFA production by Konkani consortia at 90°C (80 to 90 °C) on molasses (single strength) as carbon source during various cycles of enrichment

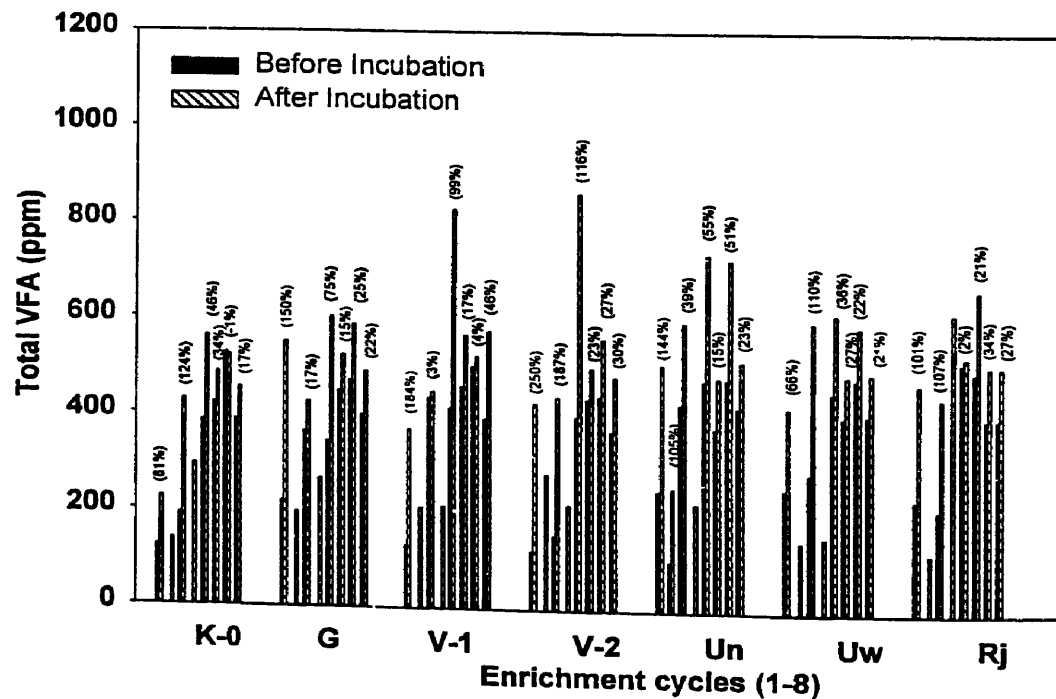


Figure 56. VFA production by Konkani consortia at 90°C (80 to 90 °C) on molasses (double strength) as carbon source during various cycles of enrichment

The characteristics of the three samples collected from Tattapani, Himachal Pradesh are shown in Table 6. Among the two substrates, glucose and molasses, at 70°C (Figures 57 to 60), molasses showed better volatile fatty acid production with the consortium T-1 and T-2 while T-3 showed enhanced VFA formation both on glucose (double strength) and molasses (single strength). At 70°C, on molasses as carbon source, both T-1 and T-2 produced propionic acid along with acetic acid, while T-3 produced only acetic acid. While acetic acid was the only product with glucose by all the three consortia.

Table 6 Characteristics of the samples collected from Tattapani, Himachal Pradesh

Sample No	Place	Source	Sample Temperature (°C)	Sample texture and colour
T-1	Tattapani	Hot water spring	45-65	Greyish- white turbid water with a mixture of grey, white and black shining sand crystals without any visible organic residues
T-2	Tattapani	Hot water spring	50-68	Greyish- white turbid water with a mixture of grey, white and black shining sand crystals without any visible organic residues
T-3	Tattapani	Hot water spring	45-68	Greyish- white turbid water with a mixture of grey, white and black shining sand crystals without any visible organic residues

At 80°C, during the five enrichment cycles, both T-1 and T-2 consortia showed increasing trend in VFA formation on molasses (double strength). A comparison between single and double strengths indicated towards suitability of using carbon sources in double strengths for higher VFA formation by all the consortia (Figures 61 to 64). Glucose (double strength) gave about 20-25% higher VFA formation compared to molasses (double strength).

Similarly, at 90°C, maximum VFA formation (Figures 65 to 68) was observed with glucose (double strength) both by T-2 and T-3 in comparison to T-1 and uninoculated control (T-0) and also both glucose (single strength) and molasses (single and double strengths).

When the different Tattapani consortia were enriched at 90°C from 80°C, only T-2 showed enhanced VFA formation during the first three enrichment cycles with glucose (single strength) as carbon source. Maximum VFA formation was observed both on glucose and molasses (double strength) compared to there single strength treatments (Figures 69 to 72).

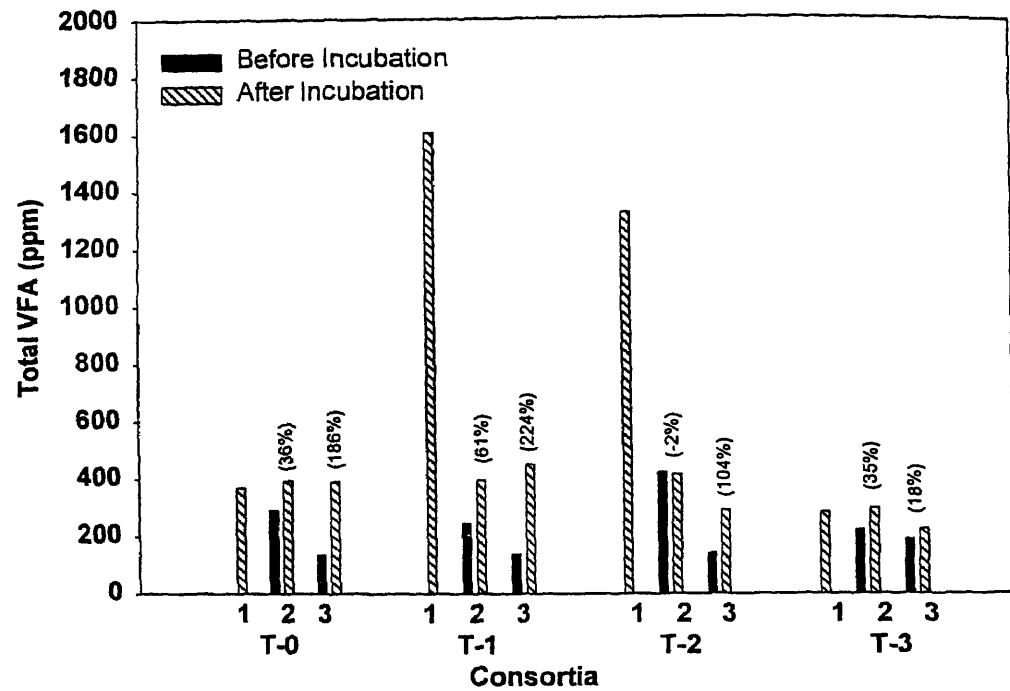


Figure 57. VFA production by Tattapani consortia at 70 °C on glucose (single strength) as carbon source during various cycles of enrichment

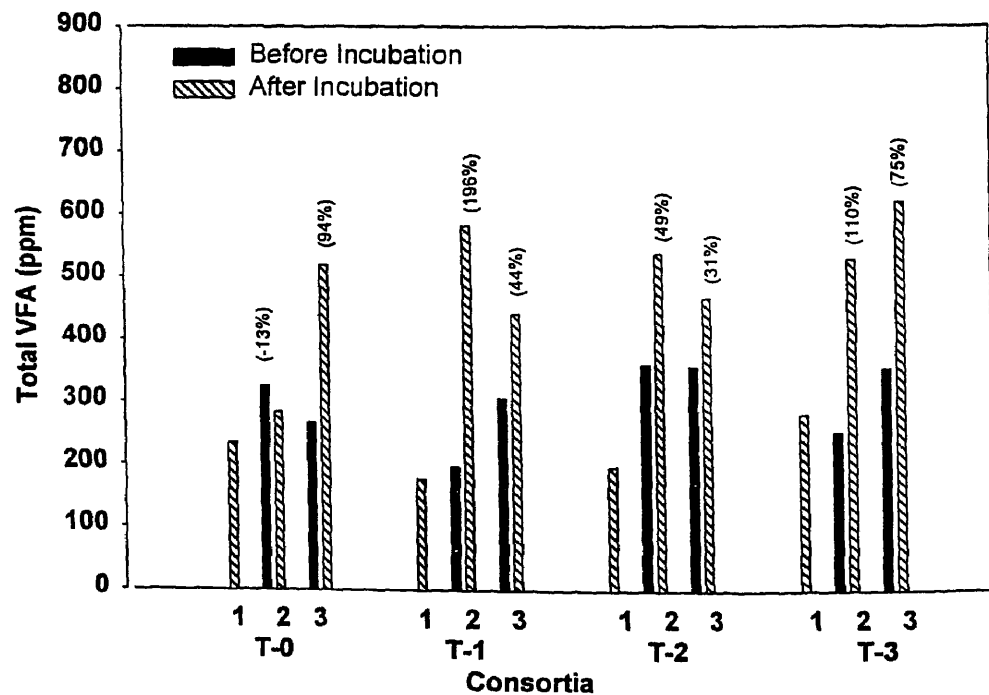


Figure 58. VFA production by Tattapani consortia at 70 °C on glucose (double strength) as carbon source during various cycles of enrichment

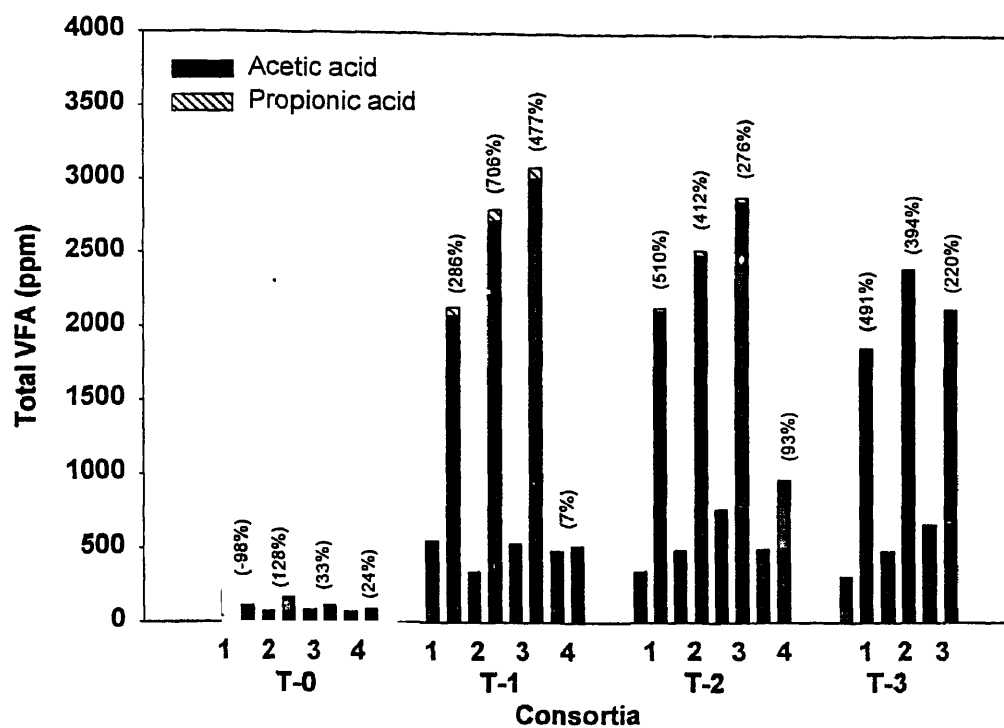


Figure 59. VFA production by Tattapani consortia at 70 °C on molasses (single strength) as carbon source during various cycles of enrichment

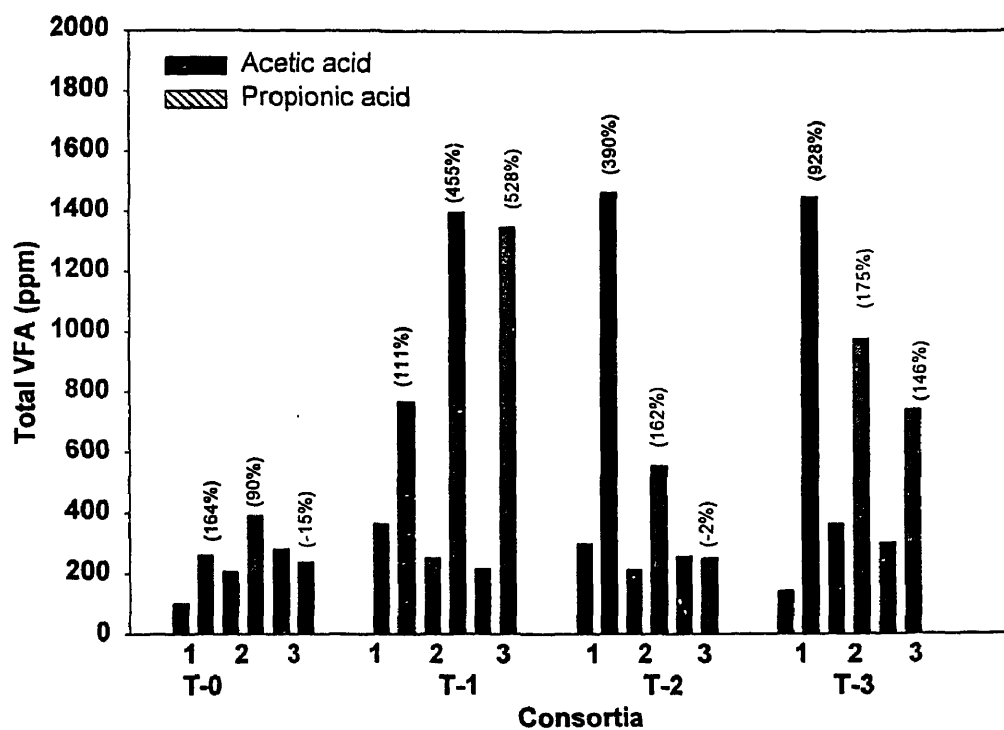


Figure 60. VFA production by Tattapani consortia at 70 °C on molasses (double strength) as carbon source during various cycles of enrichment

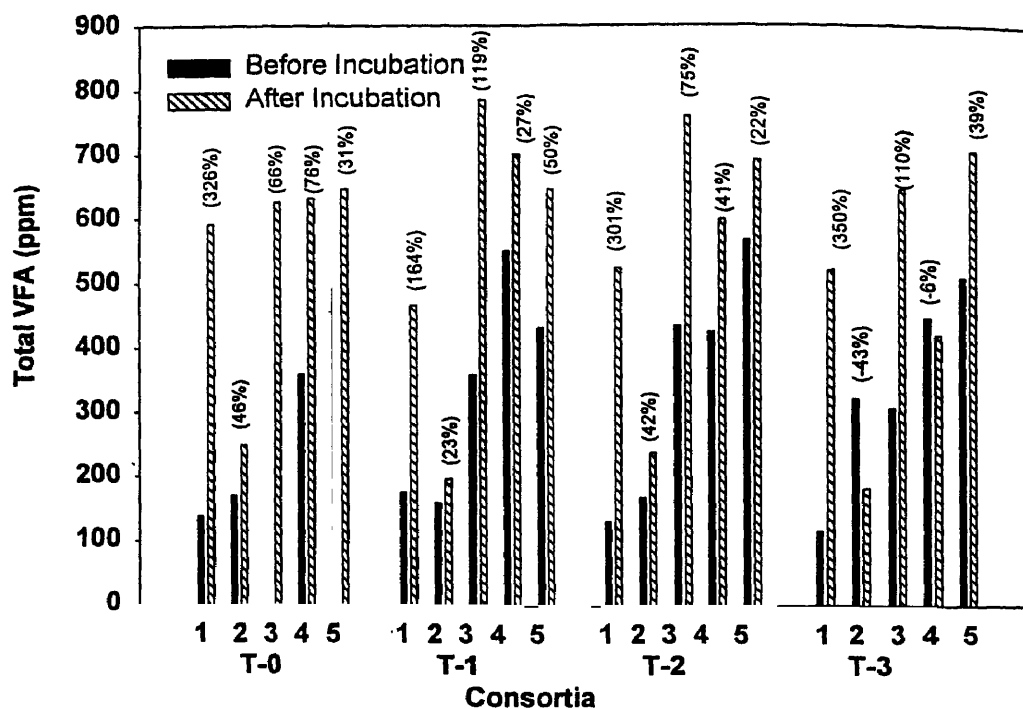


Figure 61. VFA production by Tattapani consortia at 80 °C on glucose (single strength) as carbon source during various cycles of enrichment

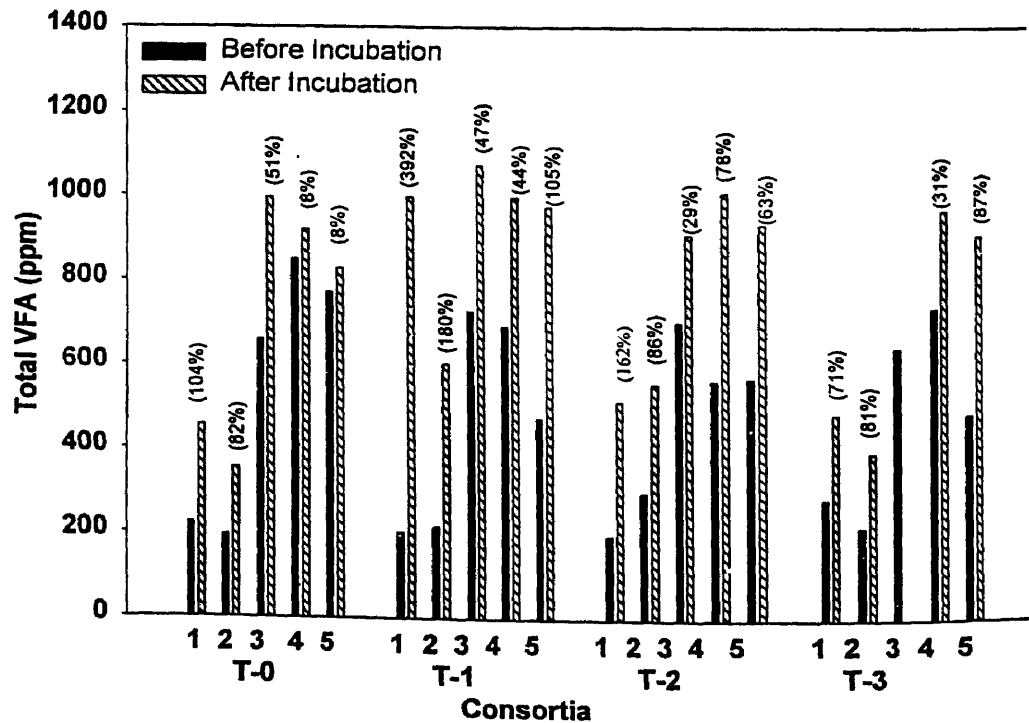


Figure 62. VFA production by Tattapani consortia at 80 °C on glucose (double strength) as carbon source during various cycles of enrichment

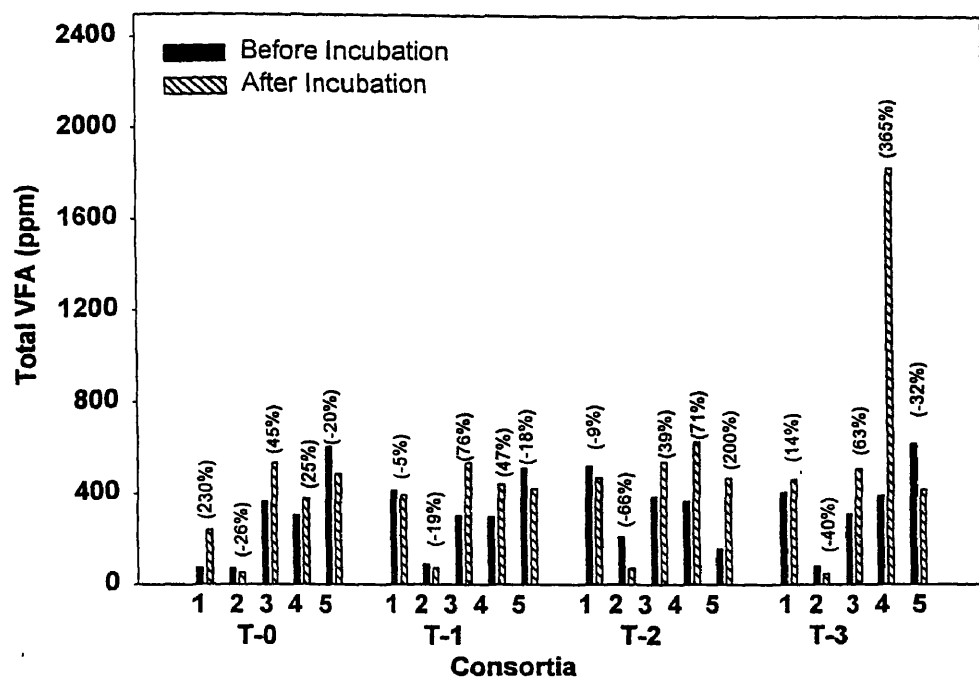


Figure 63. VFA production by Tattapani consortia at 80 °C on molasses (single strength) as carbon source during various cycles of enrichment

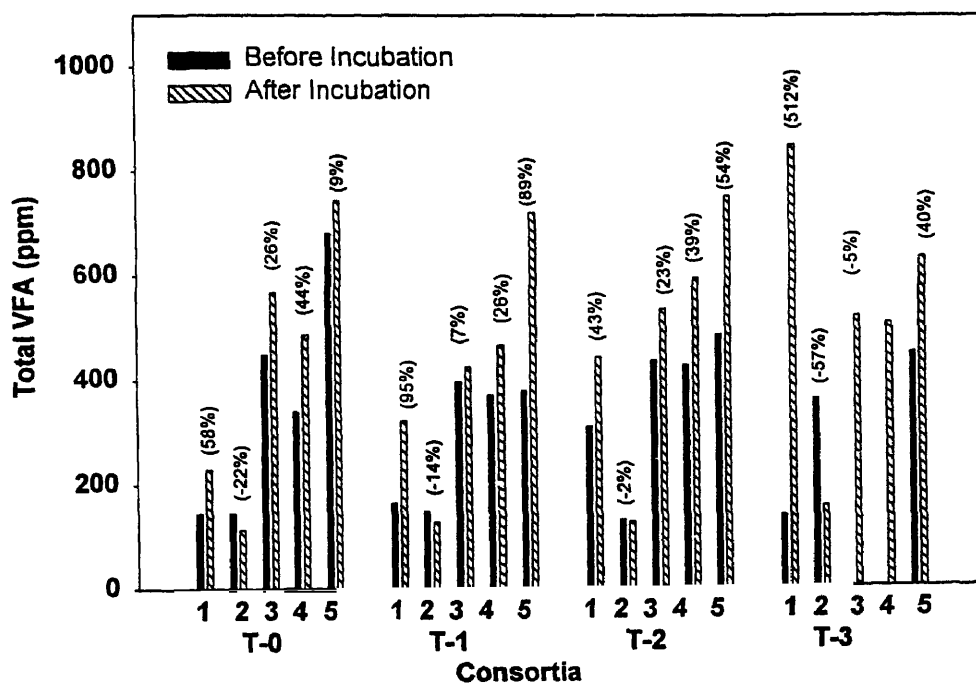


Figure 64. VFA production by Tattapani consortia at 80 °C on molasses (double strength) as carbon source during various cycles of enrichment

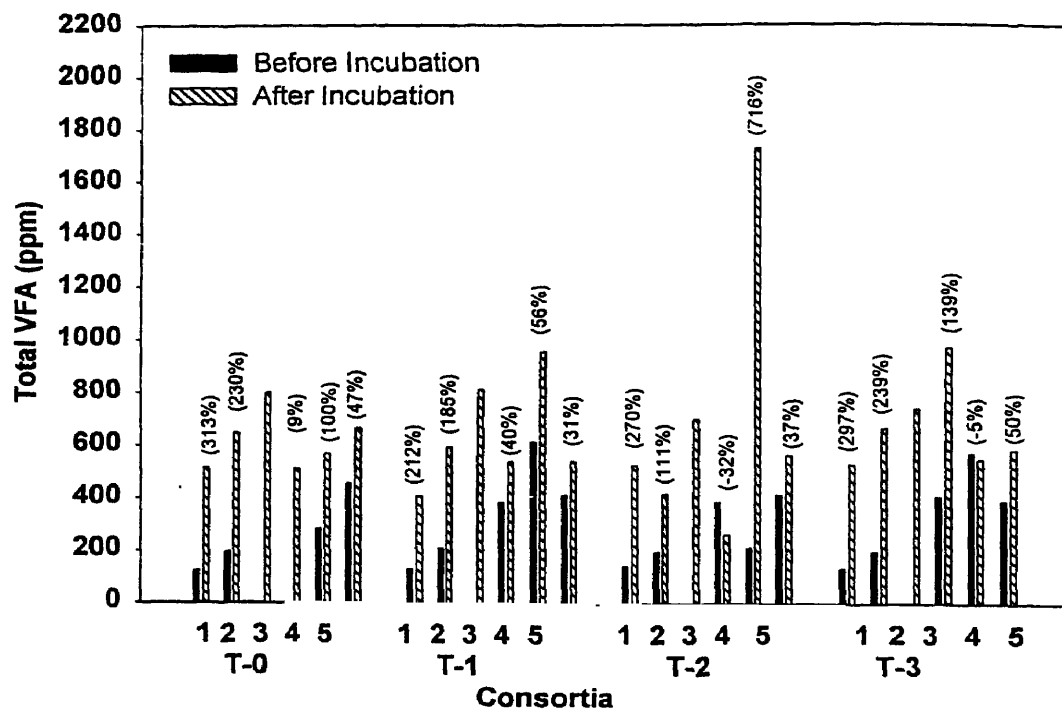


Figure 65. VFA production by Tattapani consortia at 90 °C on glucose (single strength) as carbon source during various cycles of enrichment

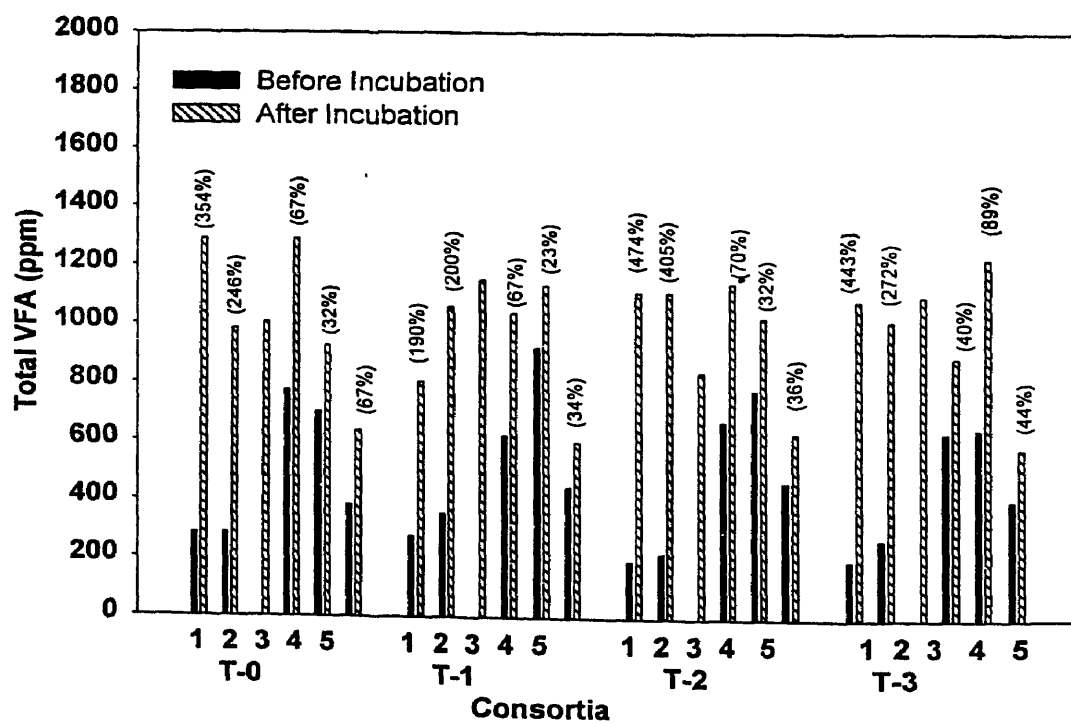


Figure 66. VFA production by Tattapani consortia at 90 °C on glucose (double strength) as carbon source during various cycles of enrichment

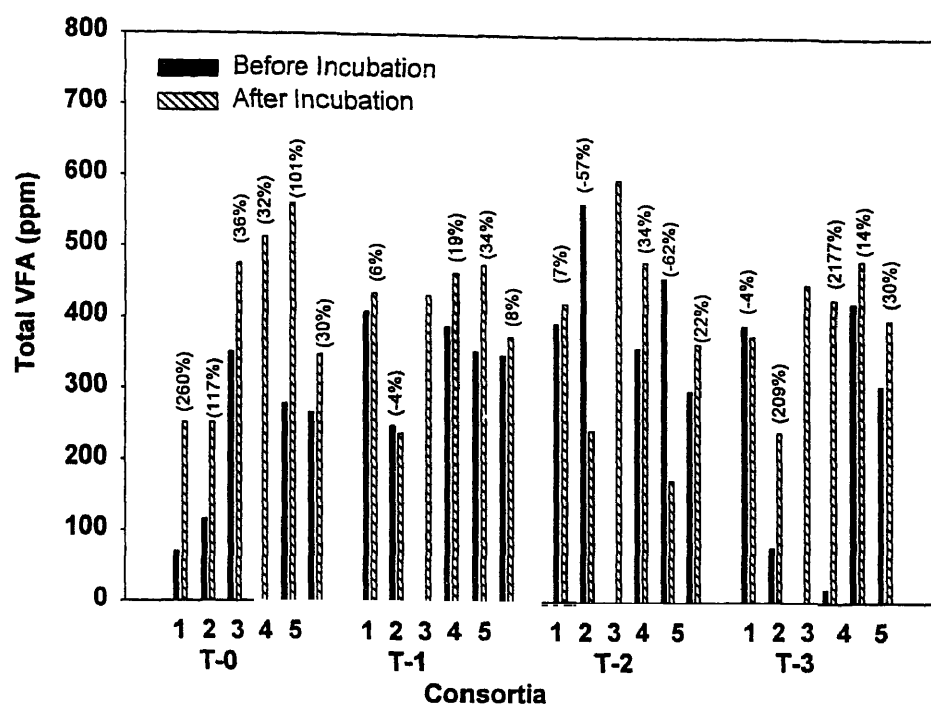


Figure 67. VFA production by Tattapani consortia at 90 °C on molasses (single strength) as carbon source during various cycles of enrichment

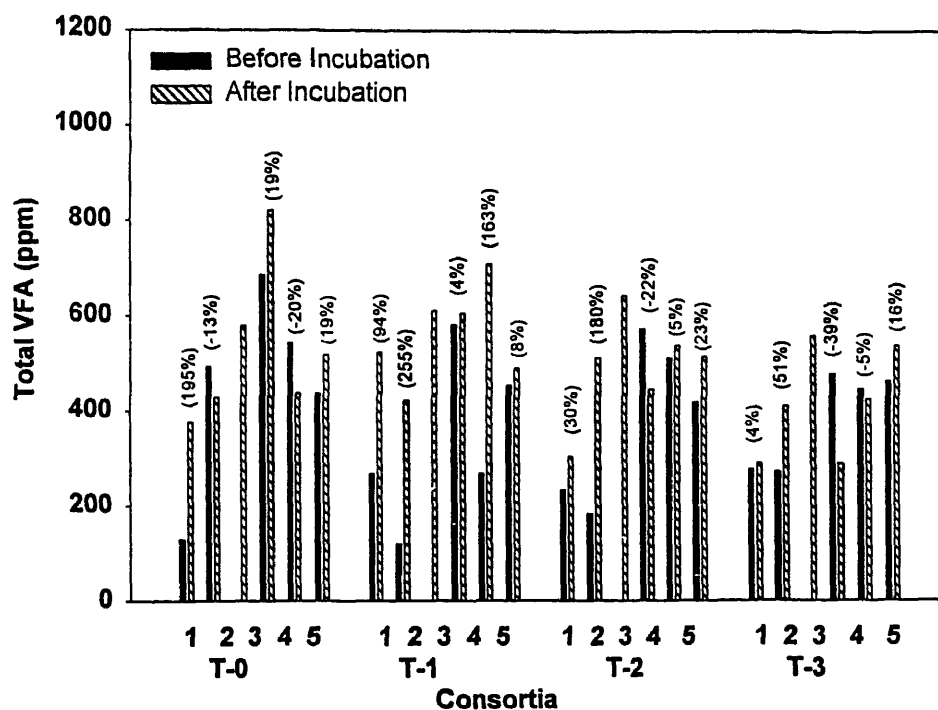


Figure 68. VFA production by Tattapani consortia at 90 °C on molasses (double strength) as carbon source during various cycles of enrichment

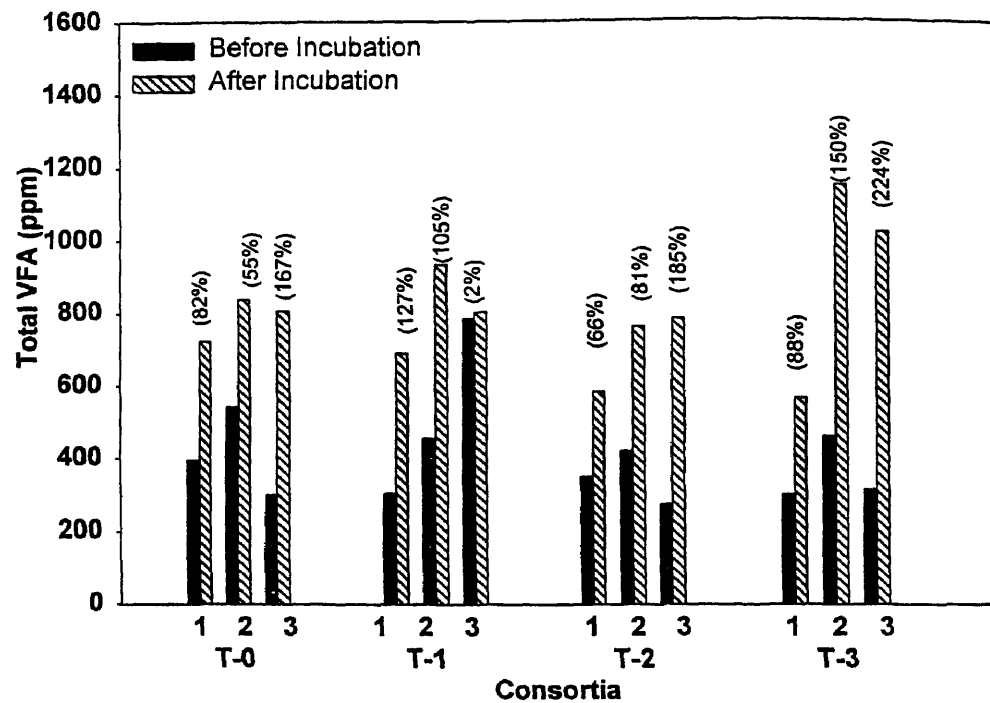


Figure 69. VFA production by Tattapani consortia at 90°C (80 to 90 °C) on glucose (single strength) as carbon source during various cycles of enrichment

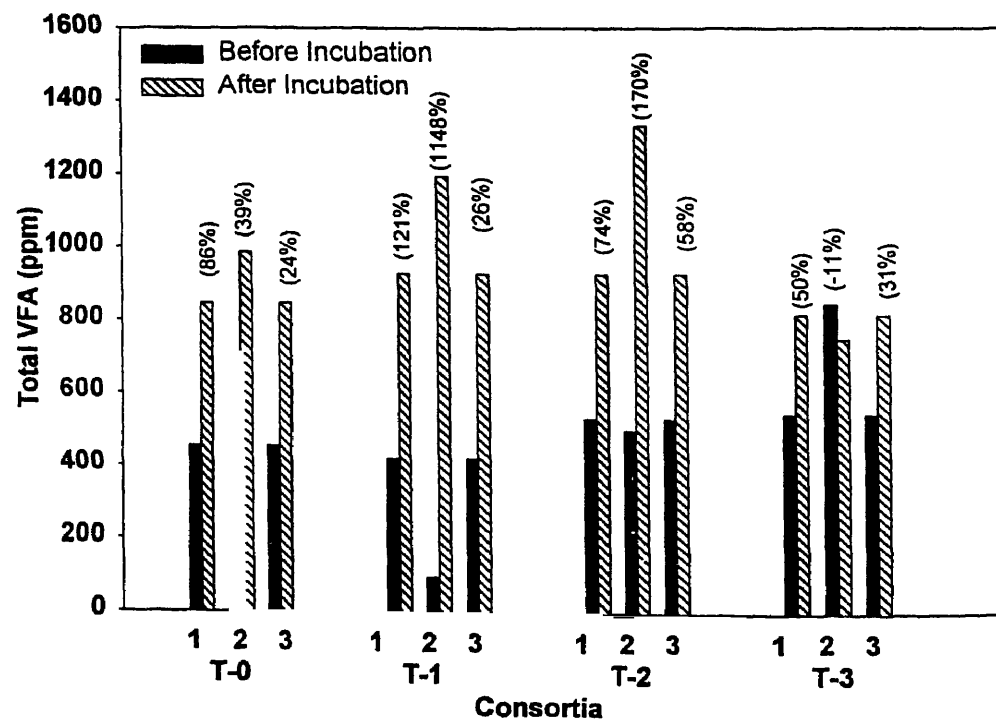


Figure 70. VFA production by Tattapani consortia at 90°C (80 to 90 °C) on glucose (double strength) as carbon source during various cycles of enrichment

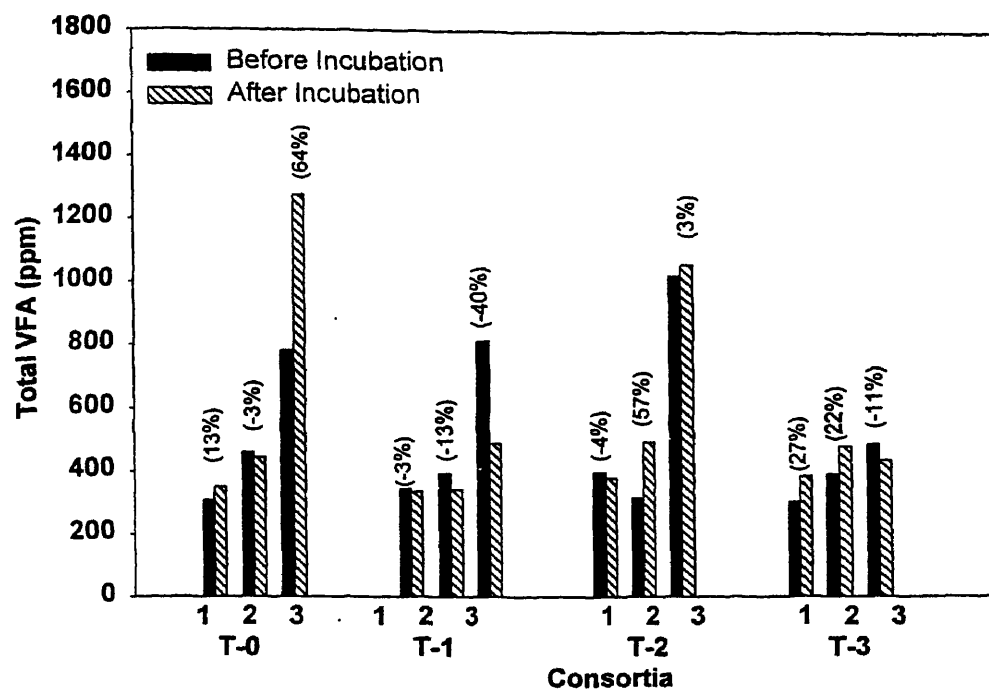


Figure 71. VFA production by Tattapani consortia at 90°C (80 to 90 °C) on molasses (single strength) as carbon source during various cycles of enrichment

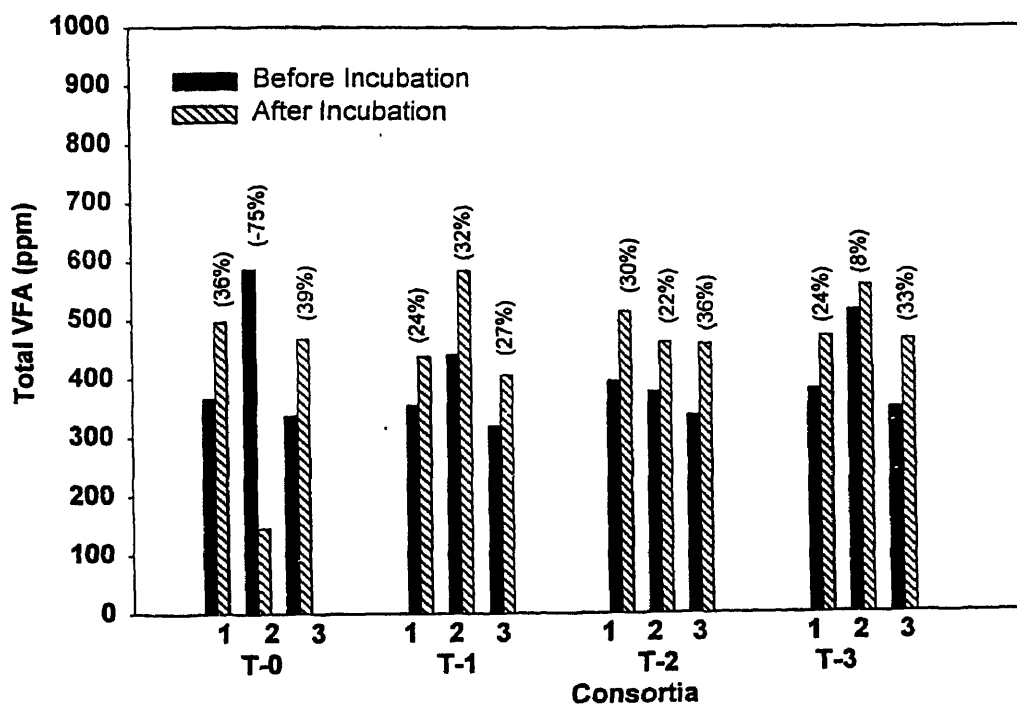


Figure 72. VFA production by Tattapani consortia at 90°C (80 to 90 °C) on molasses (double strength) as carbon source during various cycles of enrichment

MEOR trial with selected S-2 and J-1 consortia in sand packed column

Prior to use as inoculum for sand packed column trial, the consortia J-1 and S-2 were analysed for two parameters viz., the volatile fatty acid (VFA) profile and the gas production when grown on glucose and molasses (double strength). Only acetic acid was detected among the volatile fatty acids, as a product while among the gases, CO₂ was the only product detected. Consortium S-2 produced only about 705 ppm, a 218% increase over zero time (Figure 73) while consortium J-1 produced approximately 1500 ppm of acetic acid after 360 hours of incubation at 90°C, an increase of 289% over the zero time. With both the consortia, glucose (double strength) was found to be better in terms of total volatile fatty acid production compared to molasses for inoculum preparation prior to use in sand packed column trial. Consortium S-2 showed 30% CO₂ (v/v) as compared to 25% (v/v) CO₂ production by consortium J-1 (Figure 74).

With the developed inocula (both S-2 and J-1 on glucose double strength) sand packed column trial was initiated by huff and puff technique using SS cylinder (15 cm x 7 cm). The assembly was placed in a water bath maintained at 90°C. The SS assembly was filled with sand (100-200 mesh), saturated with water and then Mehsana crude oil was added at 90°C. After two days of equilibration, water was pumped from the inlet point and the water & oil coming out from the outlet was collected. The crude oil was extracted and measured gravimetrically. Subsequently the sand packed column was inoculated with the selected S-2 and J-1 consortium grown on glucose or molasses as carbon source at 90°C. After 20 days of incubation at 90°C, the column was pumped with water at 90°C, the water and oil coming out of the column was collected, and crude oil extracted and then measured gravimetrically.

In contrast to, glucose (double strength) as carbon source, which was found to be better for inoculum development prior to use in the sand packed column, during the sand packed column trial, molasses (double strength) was found to give much higher (130-200%) EOR by both the S-2 and J-1 consortia (Figures 75 & 76).

A comparison between the two consortia viz., S-2 and J-1 indicated to wards superiority of J-1 over S-2 in EOR application with both glucose and molasses as carbon sources. In case of glucose (double strength) J-1 showed 50% higher EOR over S-2 with the same carbon source, while with molasses (double strength) J-1 gave over 95% higher EOR in comparison to S-2.

Both the consortia J-1 and S-2 were tested for pathogenicity to check their suitability for final application in EOR process.

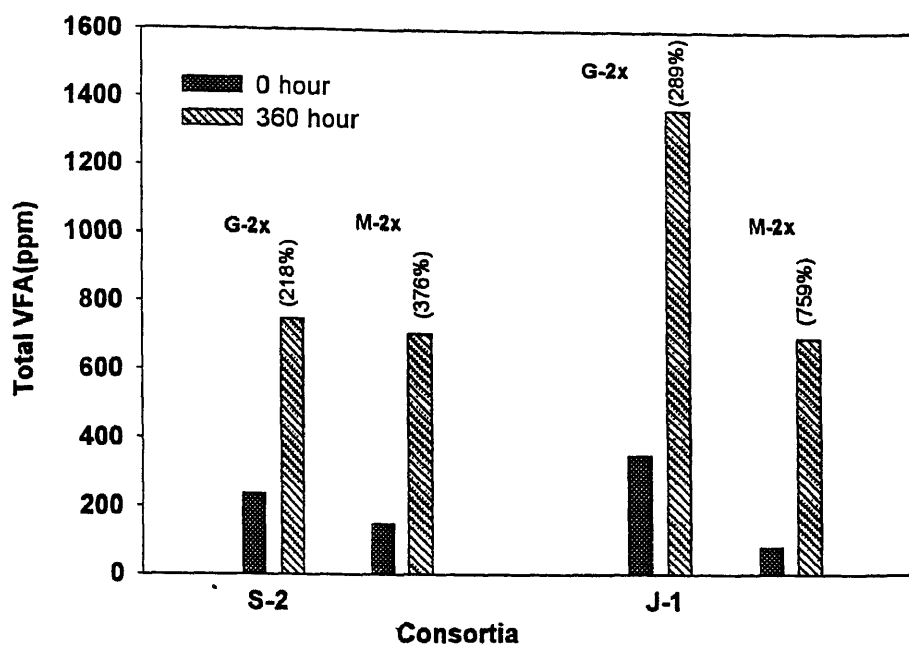


Figure 73. VFA production by selected consortia at 90 °C on glucose and molasses (double strength) for use as inoculum in sand packed column trial

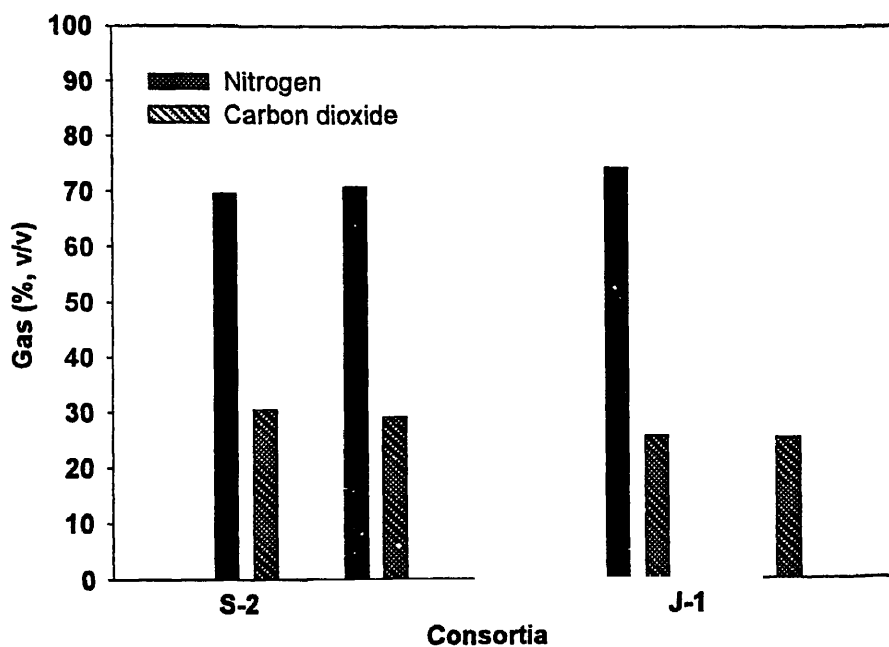


Figure 74. Production of CO₂ by selected consortia at 90 °C on glucose and molasses (double strength) for use as inoculum in sand packed column trial

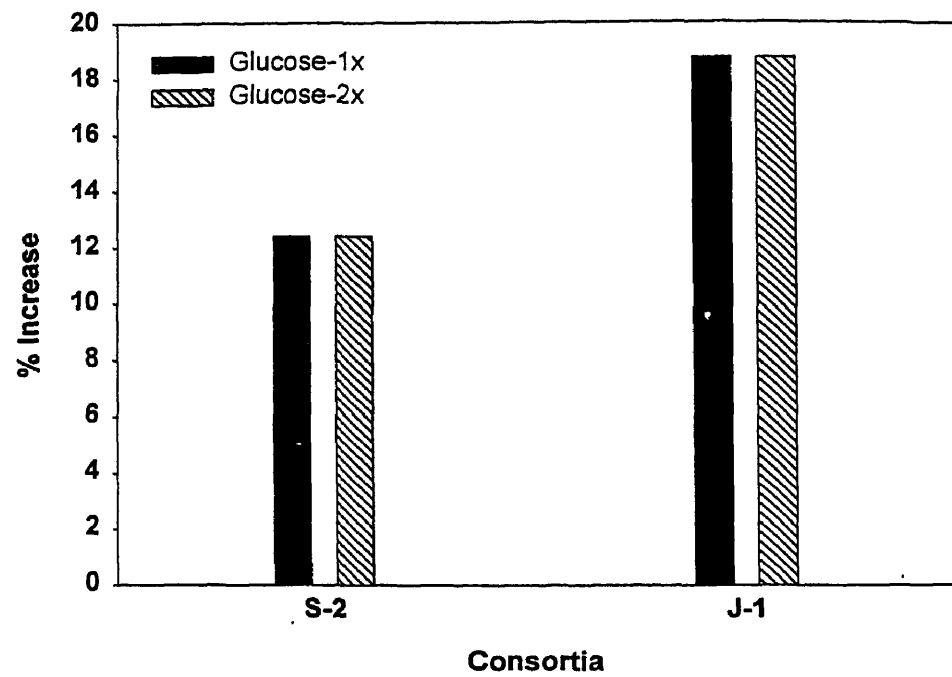


Figure 75. Enhanced oil recovery by selected consortia at 90 °C during sand packed column trial

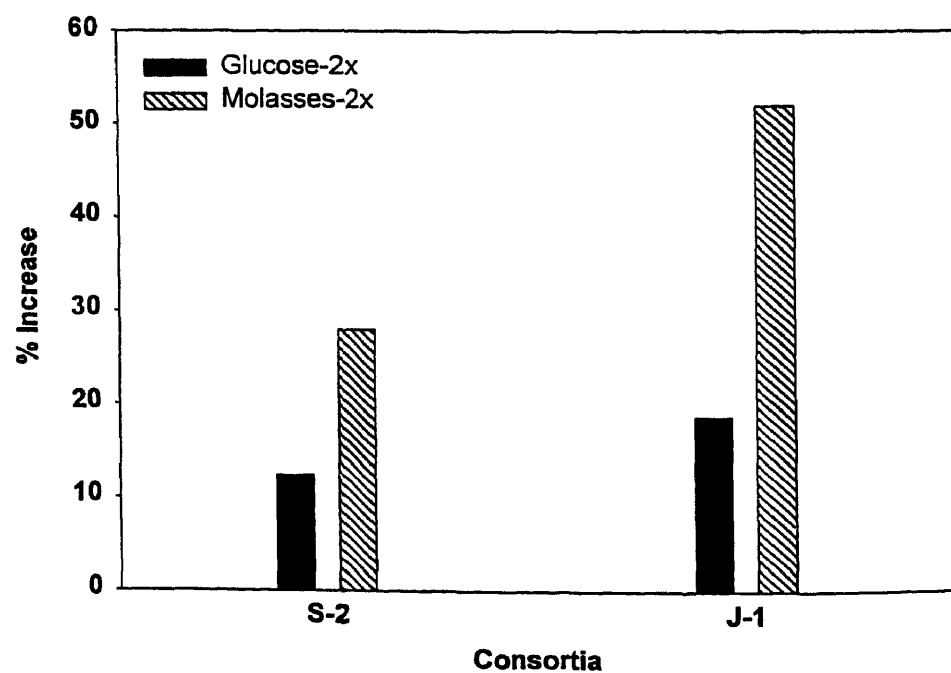


Figure 76. Enhanced oil recovery by selected consortia at 90 °C during sand packed column trial

Microscopic and physiological characteristics

Microscopic examination of the consortium J-1 and S-2 grown on glucose and molasses respectively showed that the cells of the consortia J-1 were Gram negative with both short rods and cocci and some very long rods (Plate 1). The cell of the consortia S-2 was Gram positive, small cocci and short rods and tended to occur in bunches around the substrate. The morphology of the cells of the consortium S-2 suggests bacterial cells could belong to the genus *Clostridium*.

Since J-1 was found to be pathogenic and not suitable for EOR applications, Scanning Electron Microscopy (SEM) was performed only for S-2 consortium. Electron microscopy studies on the size and the exact shape of the cells of the consortium S-2 (Plates 2 to 4) are tabulated in Table 7.

Table 7 Morphological study of thermostable anaerobic consortium S-2 (SEM)

Size of bacterial cells		Shape	Motility	Gram staining
Minimum	Maximum			
0.1 μ m	1.3 μ m	Most of cells are Cocci, few cells are small rods	Cells are motile in liquid culture	Cells are gram positive

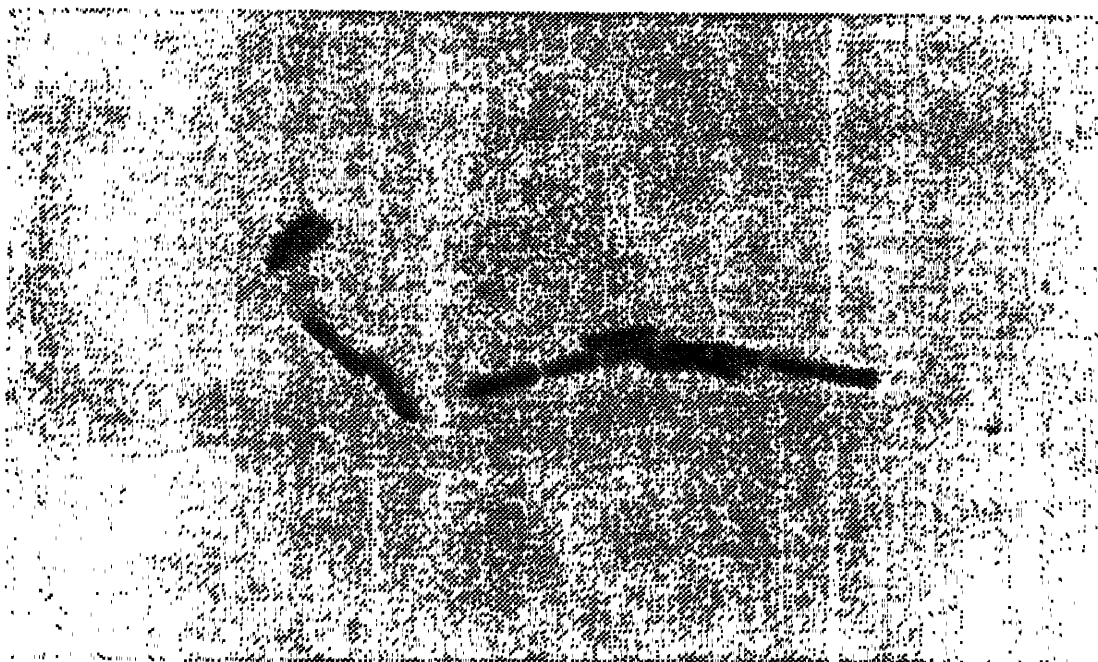


Plate 1 Light microphotograph of consortium J-1

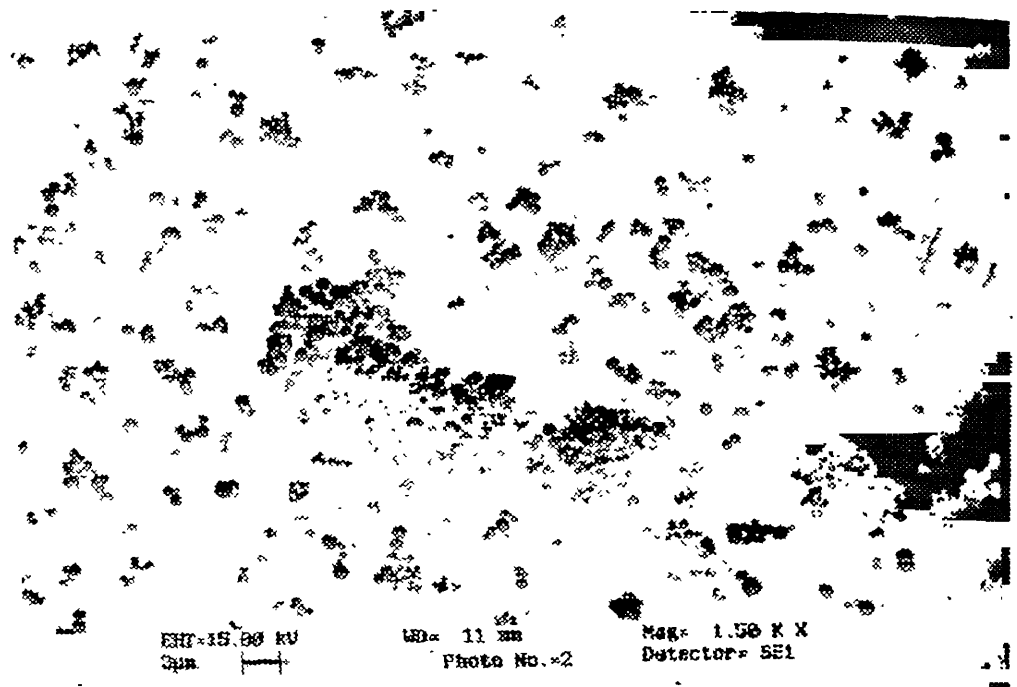


Plate 2 Scanning electron microphotograph of consortium S-2

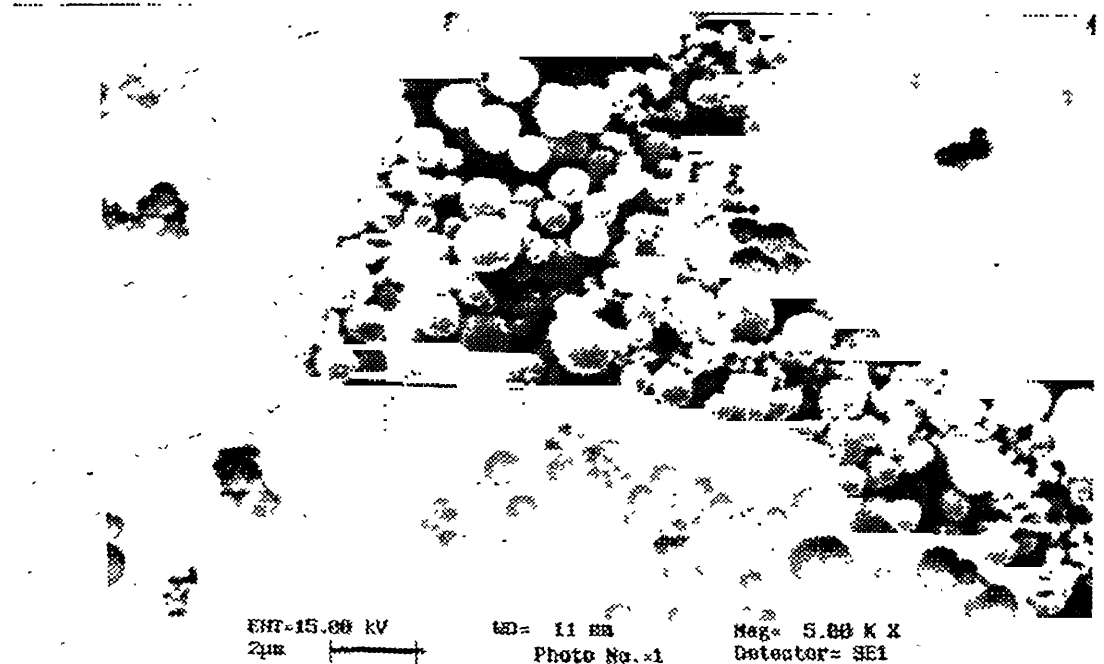


Plate 3 Scanning electron microphotograph of consortium S-2

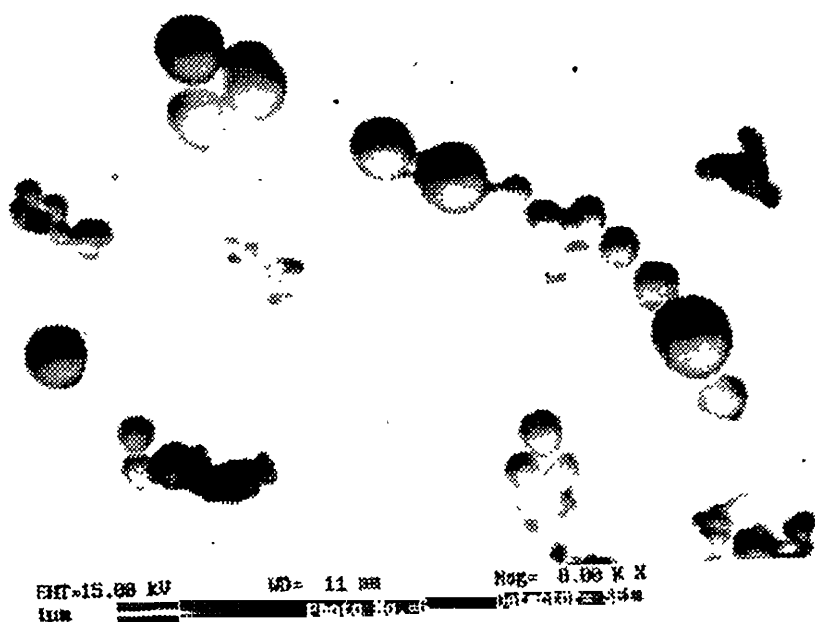


Plate 4 Scanning electron microphotograph of consortium S-2

Effect of salinity on selected S-2 consortium at 90°C

The halophilic nature of the S-2 consortium was checked using sodium chloride (0.0 to 3.0% w/v with an increment of 0.5% between the treatments) in the Balch *et al.* (1977) medium during two separate sets of experiments. In the first experiment, 0.0 to 2.0% NaCl was used in glucose (double strength) as carbon source and VFA production was found to be between 1200 to 1300 ppm at 0.0 to 1.0% salt concentrations and a decrease there after (Figure 77) after 360 hours of incubation at 90°C.

During the second experiment, a more detailed analysis of VFA formation, carbon dioxide production, and sugar utilization was carried out with respect to regular time intervals (every 48 hours) during 528 hours of incubation at 90°C on both glucose and molasses (single and double strength) as carbon sources.

The VFA production was higher with both glucose and molasses (double strength) at all salt concentrations. The maximum VFA formation was observed between 100-150 hours of incubation at all salt concentrations irrespective of the type of carbon sources and their concentrations (Figures 78 to 81). The S-2 consortium tolerated NaCl concentration upto 3.0% but optimum salt concentration for VFA production was found to be 0.5% (w/v).

The results on trend in CO₂ production (Figures 82 to 85) also confirmed the suitability of double strength carbon sources over their single strength. Invariably, CO₂ production was found to be increasing with incubation time upto a salt concentration of 2.0%.

The results on change in pH during 528 hours of incubation showed that from original pH of the medium (8.3), the maximum reduction was upto pH 5.04 with glucose (single strength) and 0.5% salt concentration (Figures 86 to 89). Contrary to VFA formation with double strength carbon sources, the maximum reduction in pH was observed on single strength glucose (5.04) and molasses (6.60).

The results on sugar utilization with respect to time over a 336 hours of incubation at 90°C (Figures 90 to 93) showed that double strength was better compared to single strength (glucose and molasses), following the trend in VFA formation. Irrespective of the carbon source and its strengths, utilization of sugars in first 48 hours of incubation increased with increasing salt concentrations upto 1.5 to 2.0% . However, during the 144 hours of incubation, a reverse trend was observed, with maximum utilization of sugars occurring with lower concentrations of salts from 2.0 to 0.0%. With most of the salt concentrations, during 336 hours of incubation, 40-65% utilization of sugars was observed.

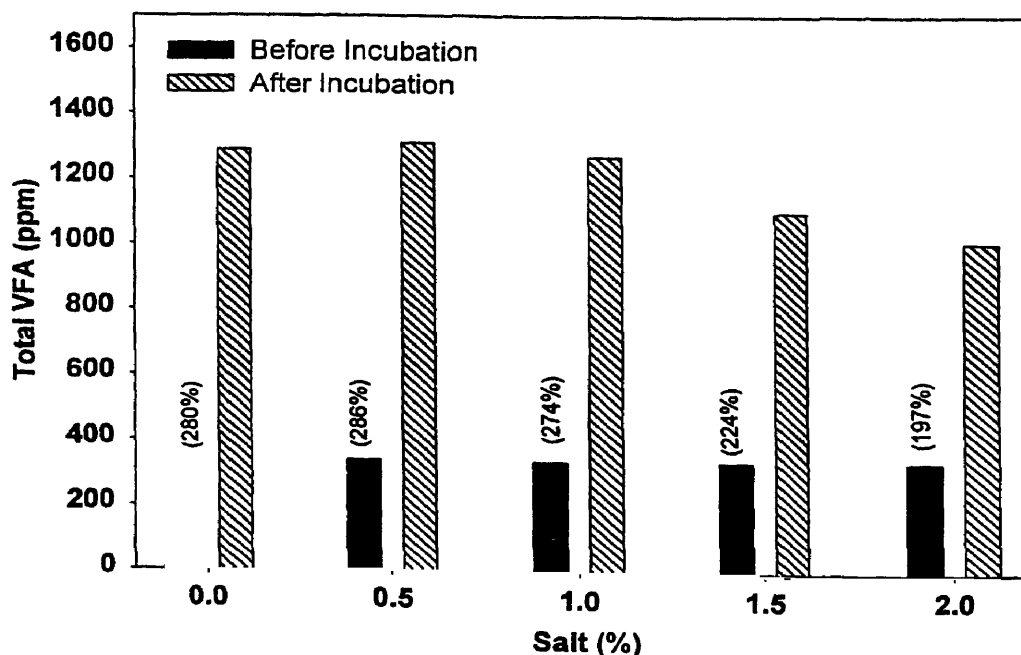


Figure 77. Effect of salinity on VFA production by S-2 consortium at 90 °C

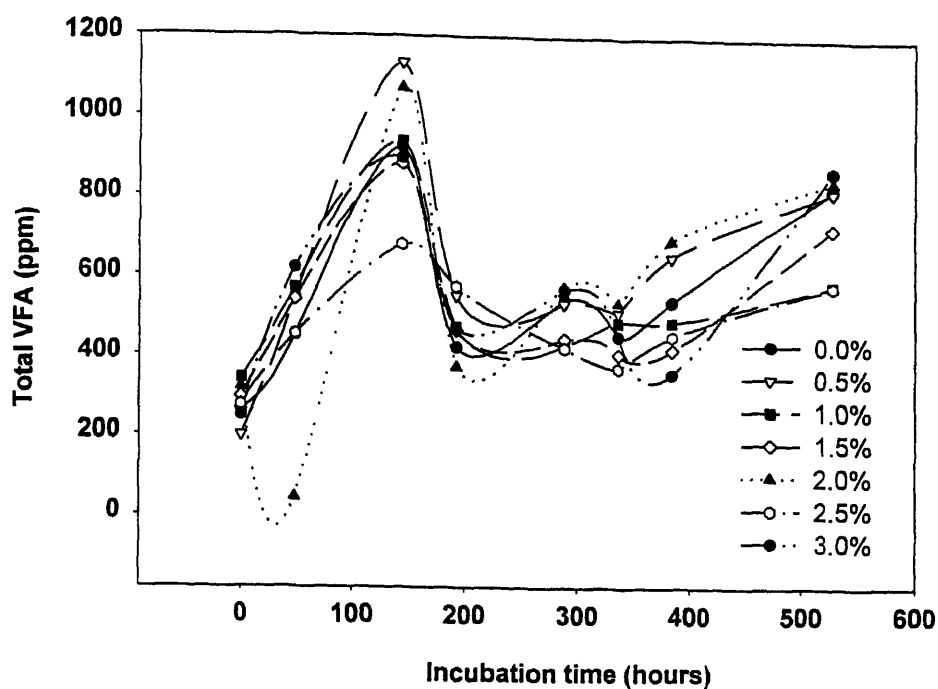


Figure 78. Effect of salinity on change in VFA production on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

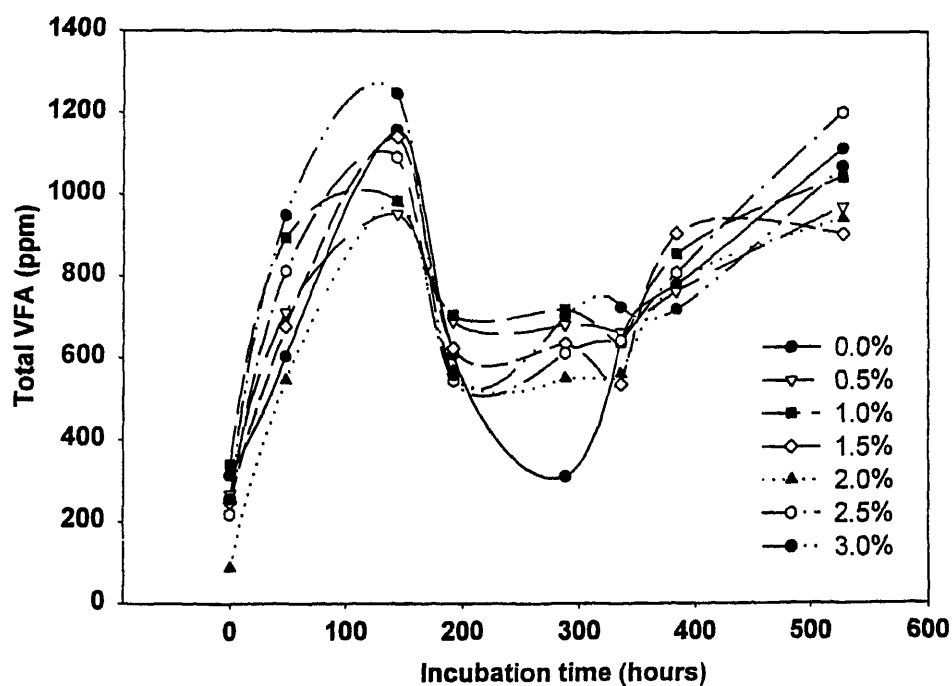


Figure 79. Effect of salinity on change in VFA production on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

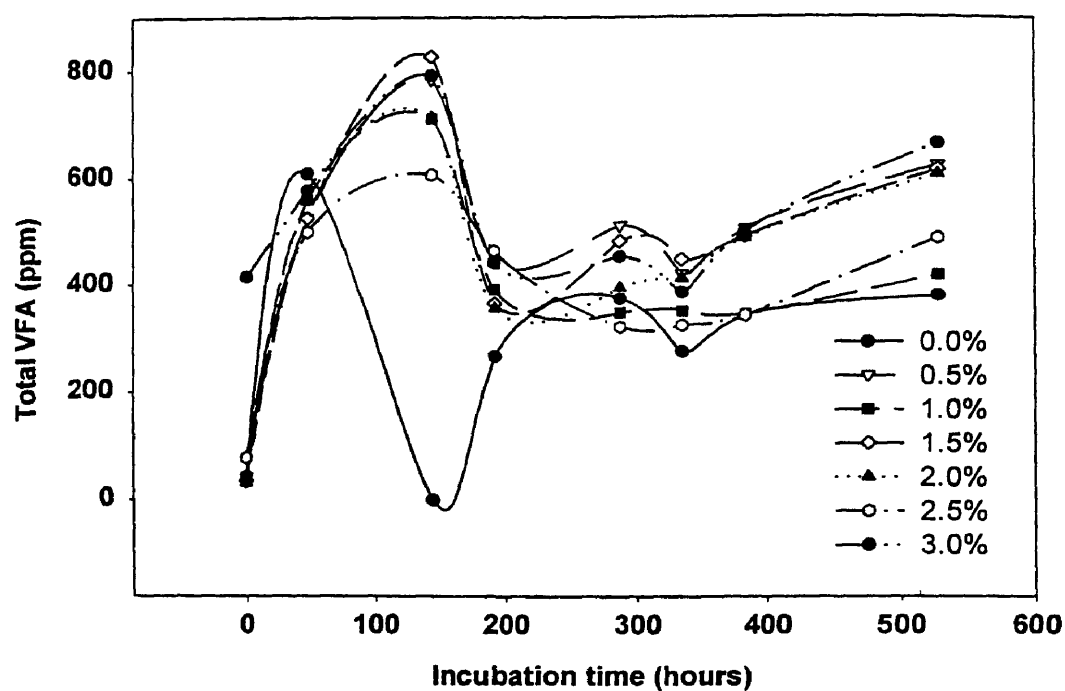


Figure 80. Effect of salinity on change in VFA production on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

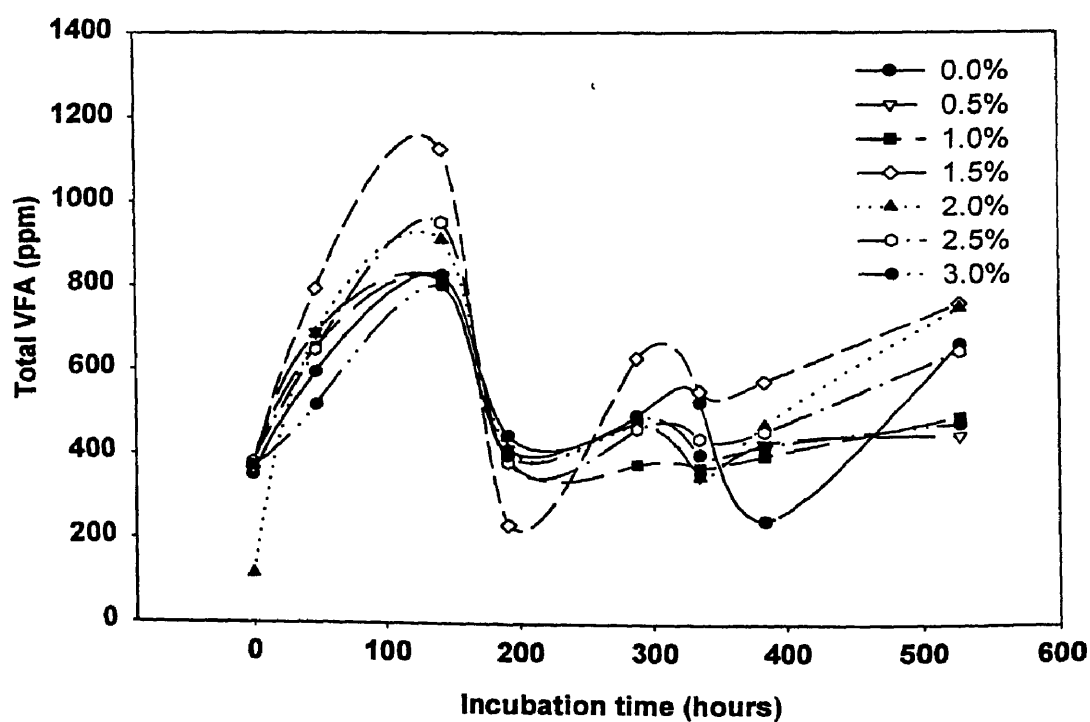


Figure 81. Effect of salinity on change in VFA production on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

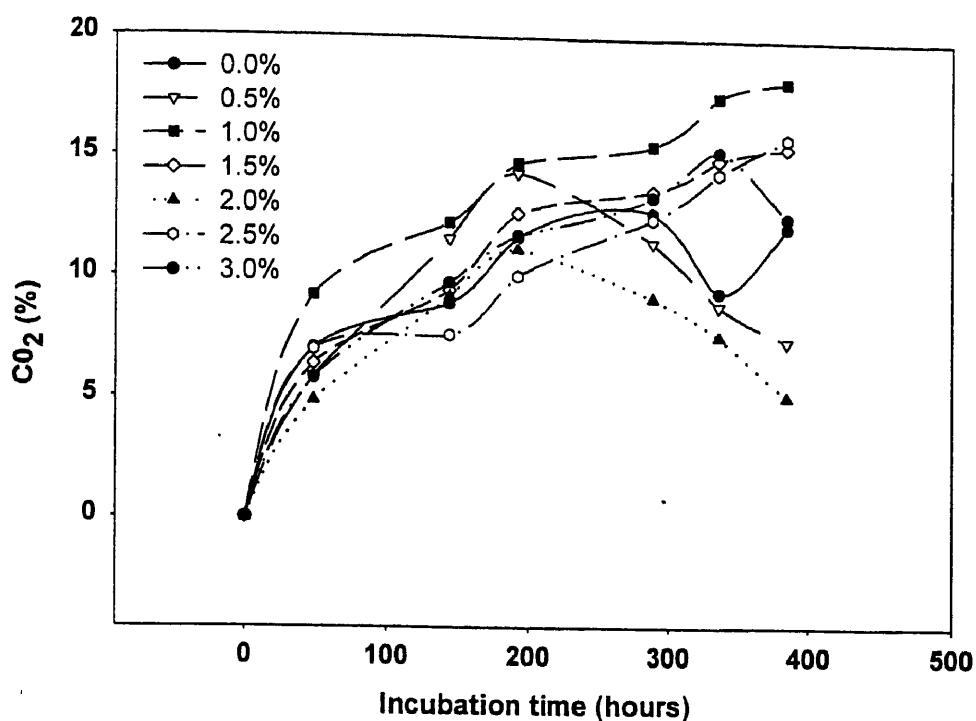


Figure 82. Effect of salinity on change in CO_2 production on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

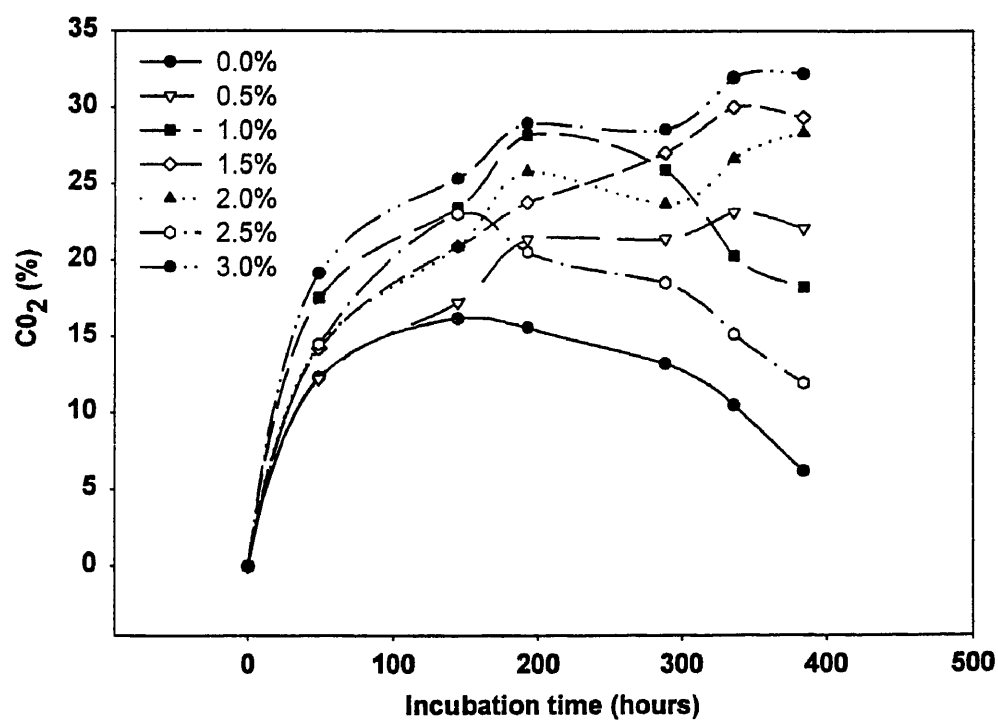


Figure 83. Effect of salinity on change in CO_2 production on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

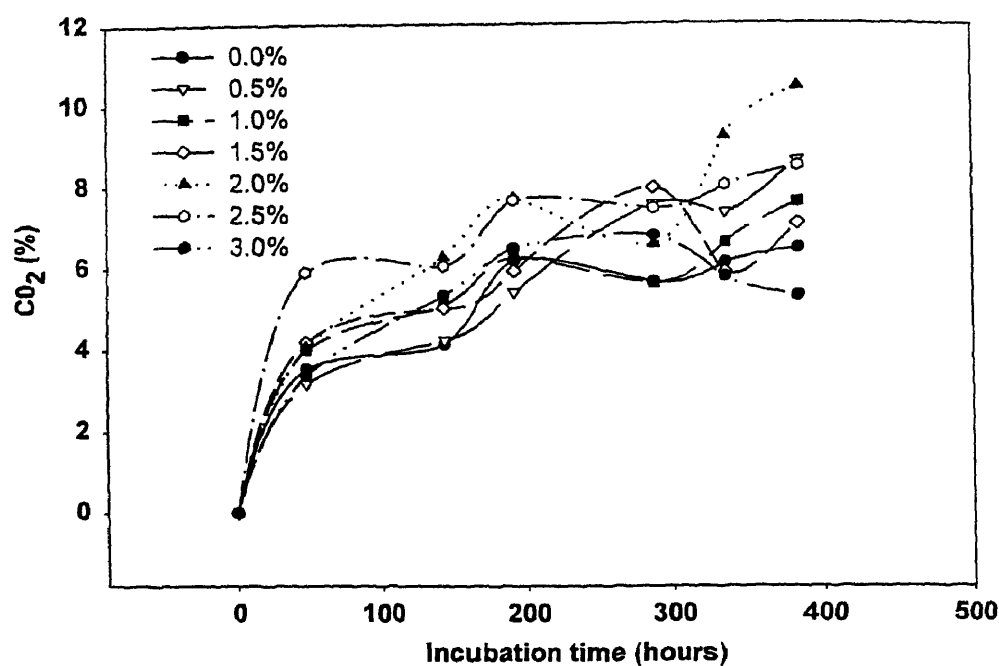


Figure 84. Effect of salinity on change in CO₂ production on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

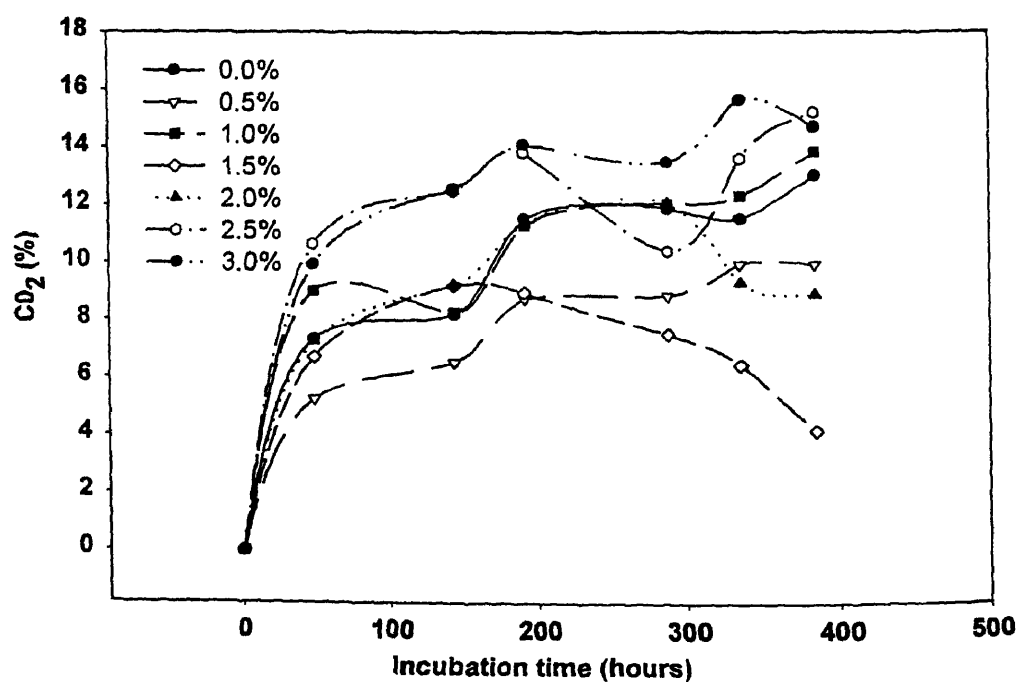


Figure 85. Effect of salinity on change in CO₂ production on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

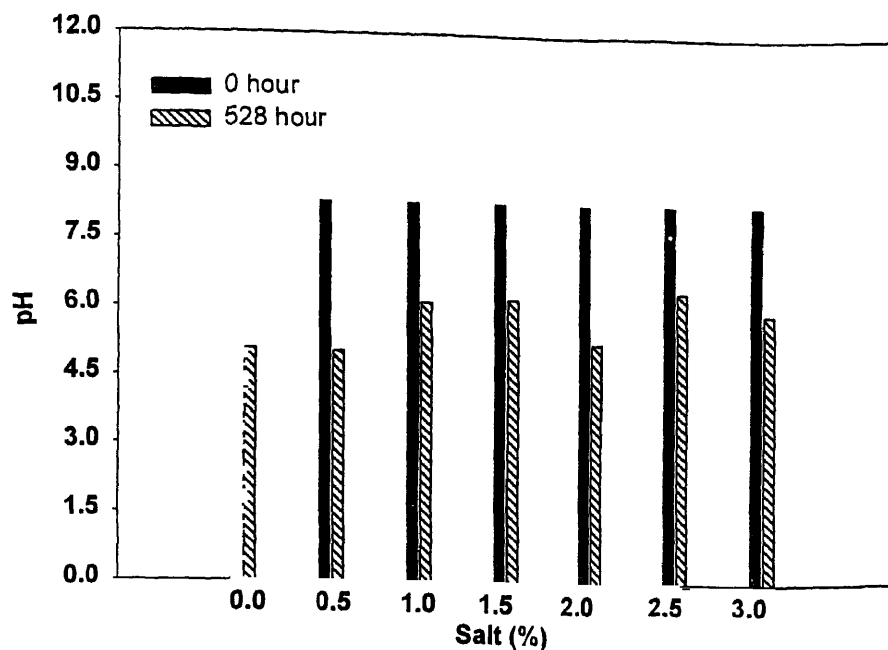


Figure 86 . Effect of salinity on change in pH on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

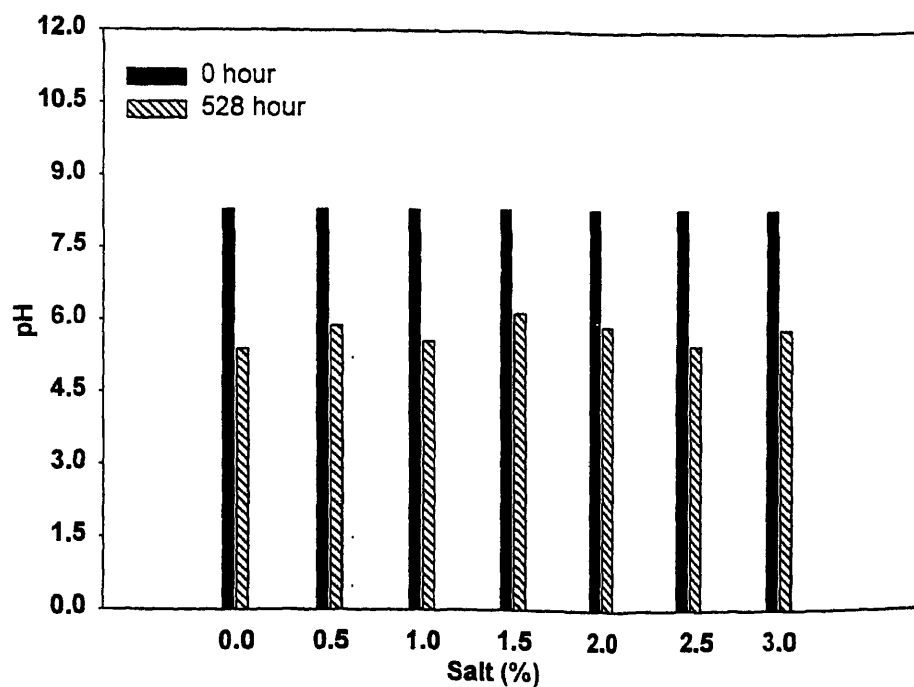


Figure 87 . Effect of salinity on change in pH on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

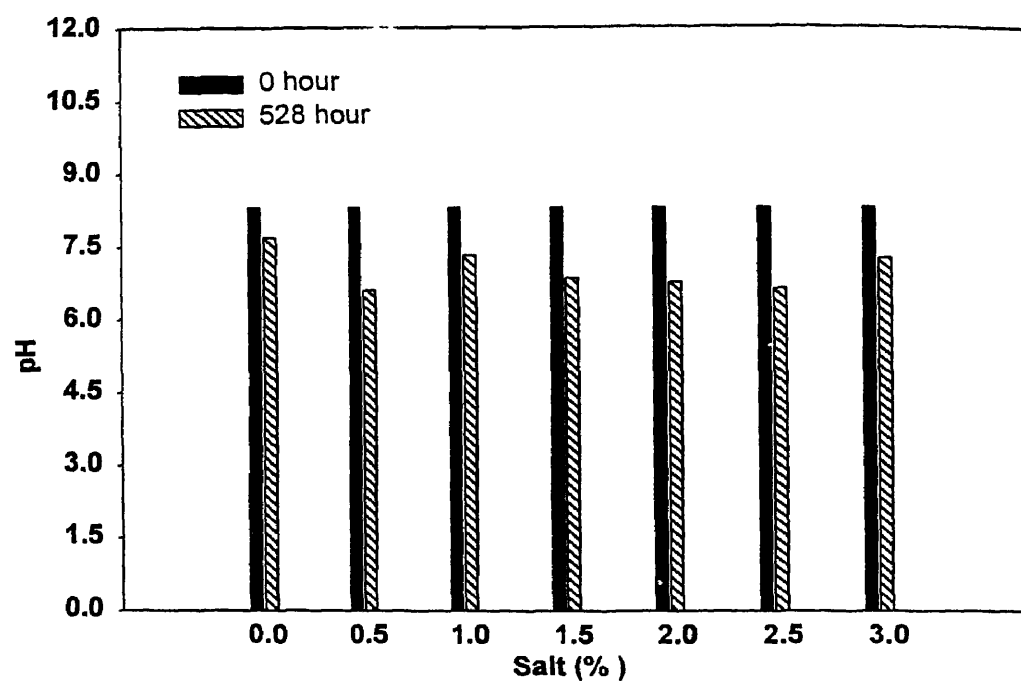


Figure 88 . Effect of salinity on change in pH on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

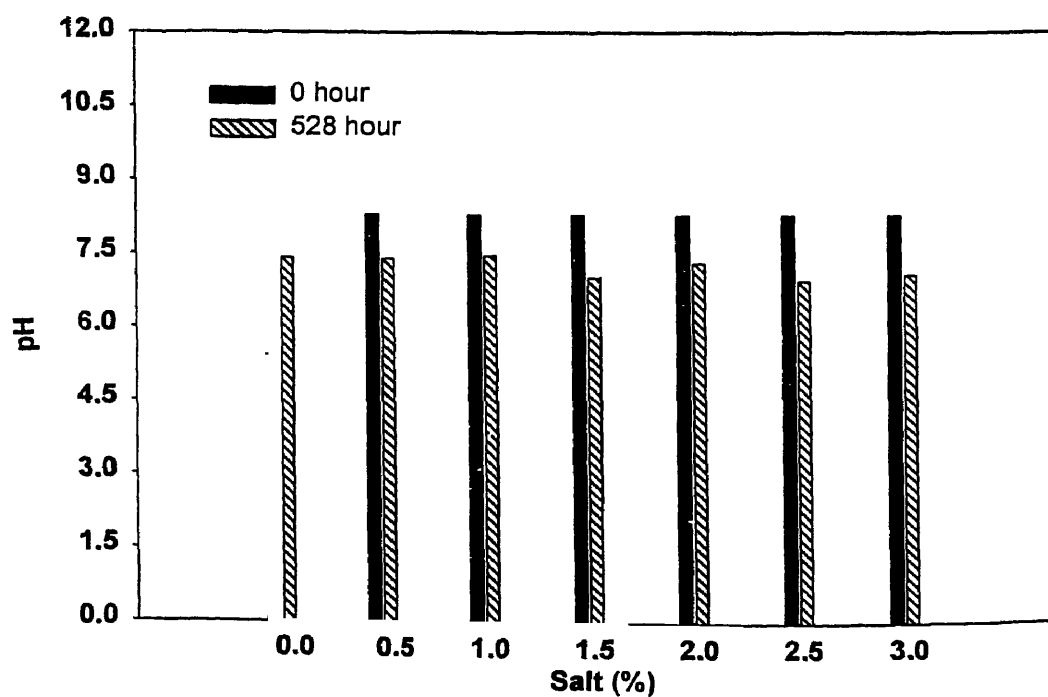


Figure 89 . Effect of salinity on change in pH on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

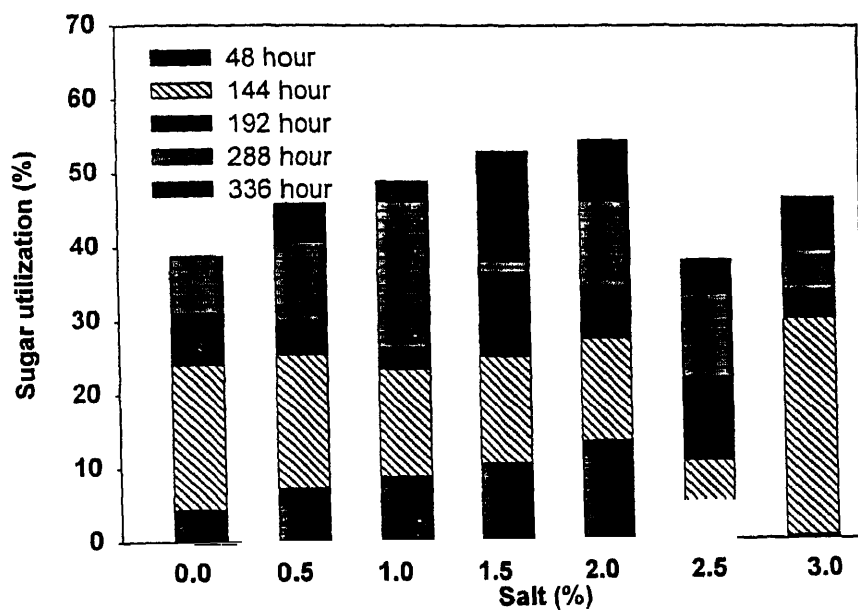


Figure 90. Effect of salinity on total sugars utilization on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

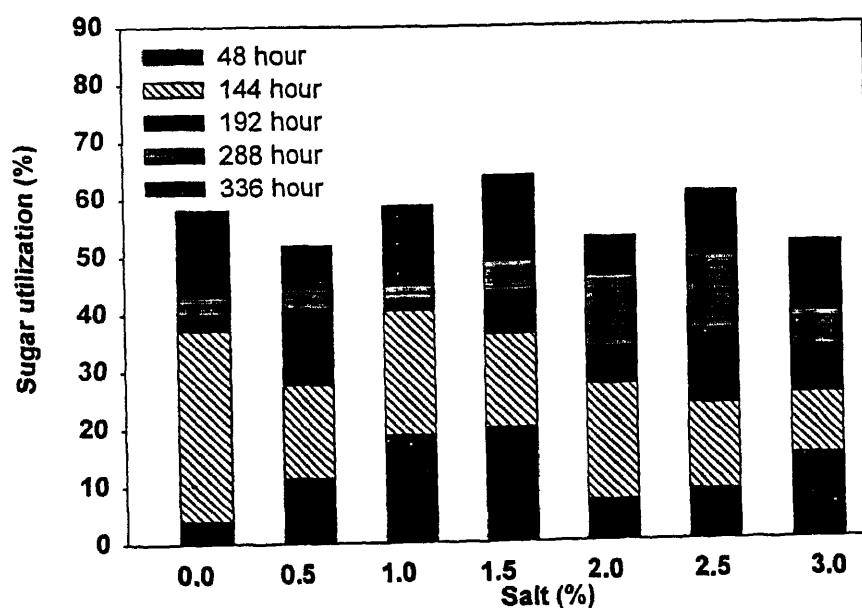


Figure 91. Effect of salinity on total sugars utilization on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

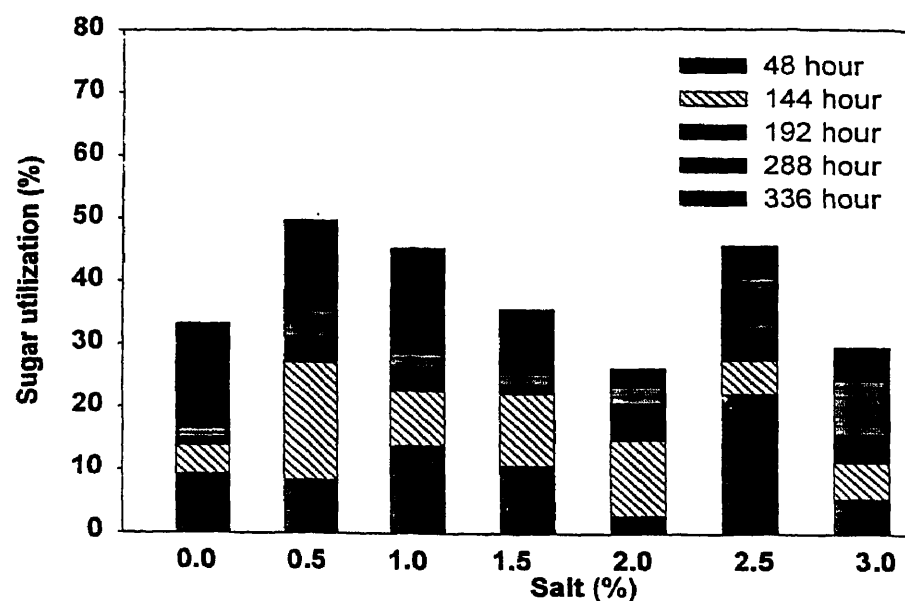


Figure 92. Effect of salinity on total sugars utilization on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

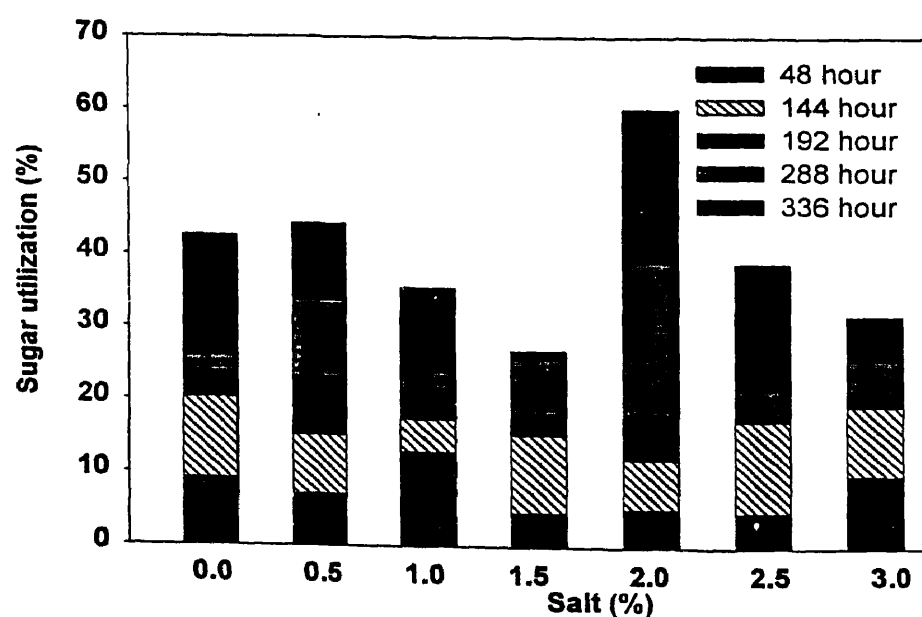


Figure 93. Effect of salinity on total sugars utilization on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

Effect of pH on selected S-2 consortium at 90°C

Adjusting the pH (4.0 to 8.5) of the Balch *et al.* (1977) medium during two separate sets of experiments checked the pH tolerance of the S-2 consortium. In the first experiment, pH was adjusted between 6.0 to 8.5 (with increment of 0.5 between the treatments) and was used in glucose (double strength) as carbon source and VFA production was found to be between 1200 to 1300 ppm between pH 6.0 to 7.5 and a decrease there after (Figure 94) during 360 hours of incubation at 90°C.

During the second experiment, a more detailed analysis of VFA formation, carbon dioxide production, and sugar utilization was carried out with respect to regular time intervals (every 48 hours) during 528 hours of incubation at 90°C on both glucose and molasses (single and double strength) as carbon sources. The pH of the medium was adjusted between 4.0 to 6.0 while maintaining inoculated and un-inoculated controls, which originally had a pH of 8.3.

The VFA production was higher with both glucose and molasses (double strength) and at pH 8.3. The maximum VFA formation was observed between 100-150 hours, irrespective of the type of carbon sources and their concentrations (Figures 95 to 98) with pH 8.3. The S-2 consortium tolerated pH upto 8.3 for VFA production. At pH 4.0 and 5.0, drastic reduction in VFA formation was observed, with both glucose and molasses (single and double strengths). In un-inoculated control (pH 8.3), thermo-chemical VFA formation was also observed, which was found to be more or less equal to S-2 inoculated treatment at pH 8.3.

The results on trend in CO₂ production (Figures 99 to 102) also confirmed the suitability of double strength carbon sources over their single strength. Again both S-2 inoculated and un-inoculated control (pH 8.3) produced the maximum quantity of CO₂. An inhibition in CO₂ production was observed with lowering of pH.

The results on change in pH during 528 hours of incubation showed that from original pH of the medium (8.3), the maximum reduction was upto pH 3.09 with glucose (double strength) and pH 8.3. (Figures 103 to 106). The maximum reduction in pH was observed with double strength glucose, irrespective of pH.

The results on sugar utilization with respect to time over a 336 hours of incubation at 90°C (Figures 107 to 110) showed no definite trend in sugar utilization at various pH. With glucose as carbon source, both S-2 inoculated and un-inoculated control (pH 8.3) gave highest sugars utilization. While with molasses again S-2 inoculated (pH 8.3) showed the maximum reduction.

Among all the treatments, S-2 inoculated (pH 8.3) with glucose (single strength) as carbon source gave the highest utilization (74%). In all the cases, however, more or less a similar utilization of sugars was also observed with uninoculated control (pH 8.3) which resulted in substantial formation of VFA thermo-chemically.

In general, during the development of anaerobic, thermophilic, halophilic bacterial consortium at 90°C, irrespective of the experimental conditions, a very high quantity of VFA, and CO₂ formation due to thermo-chemical changes has been observed, sometimes even equal or more than the inoculated ones. This type of changes were also observed at lower temperatures of incubation such as 70°C, but were found to be less prominent than the inoculated ones. During one of the experiments, we tried to minimize such thermo-chemical formation of the products in the medium by adding filter-sterilized glucose and molasses after autoclaving and prior to inoculation at higher temperatures. But our experience showed that 70-80% of such changes takes place during incubation at high temperature and only about 20-30% changes occur during the autoclaving of the medium contents.

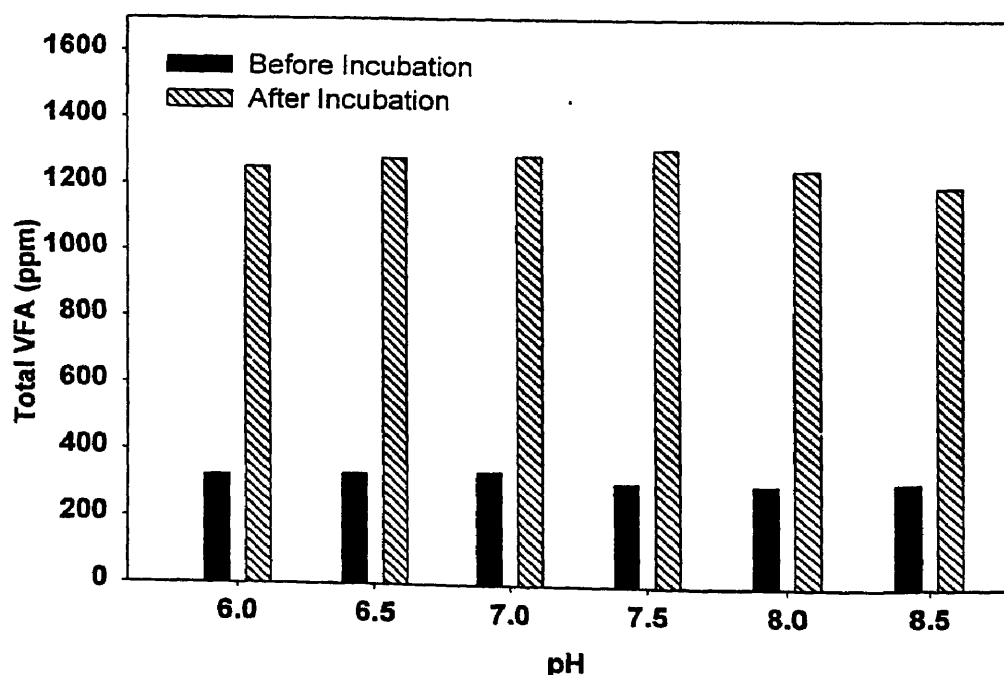


Figure 94. Effect of pH on VFA production by S-2 consortium at 90 °C

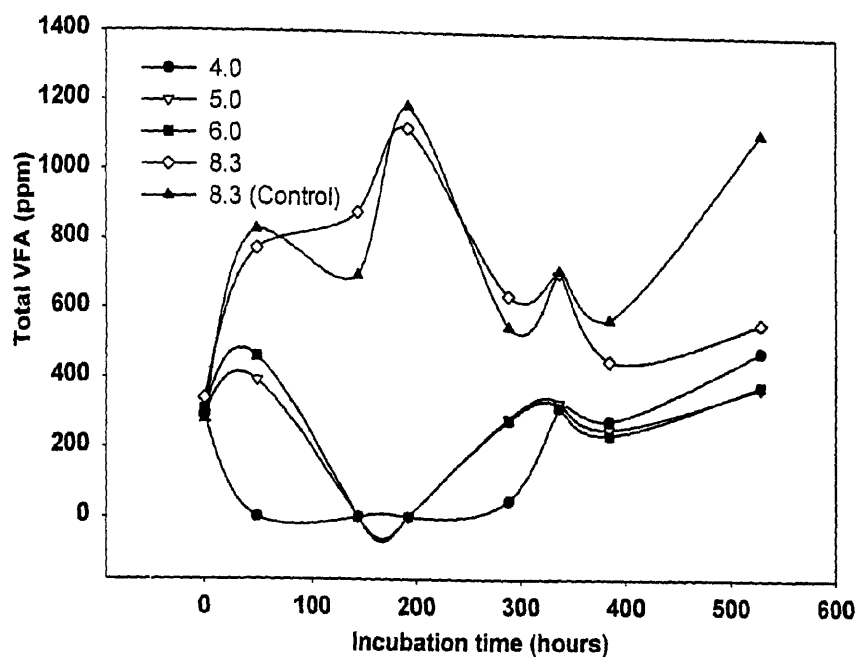


Figure 95. Effect of pH on change in VFA production on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

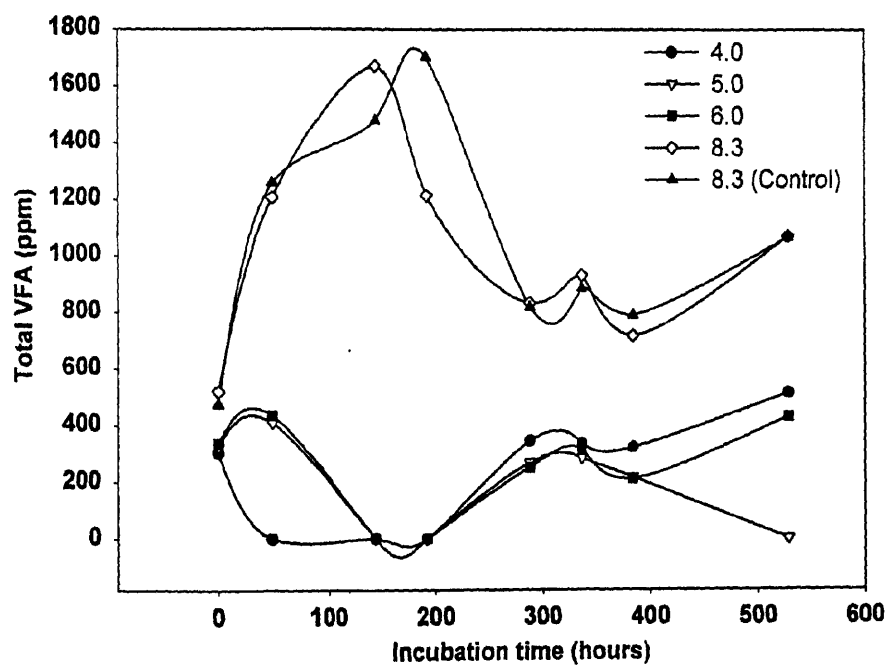


Figure 96. Effect of pH on change in VFA production on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

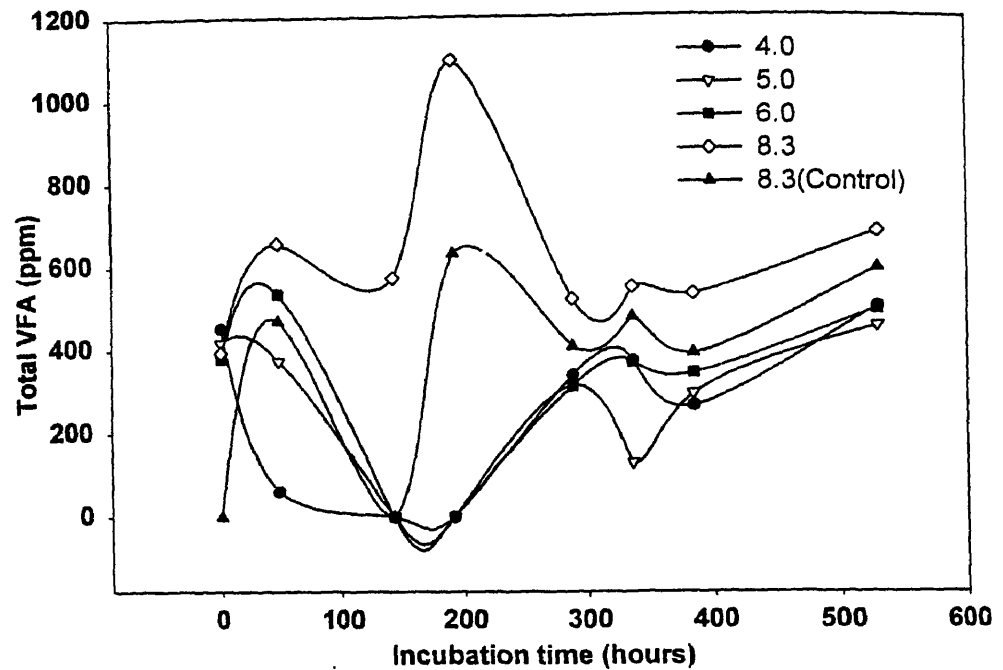


Figure 97. Effect of pH on change in VFA production on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

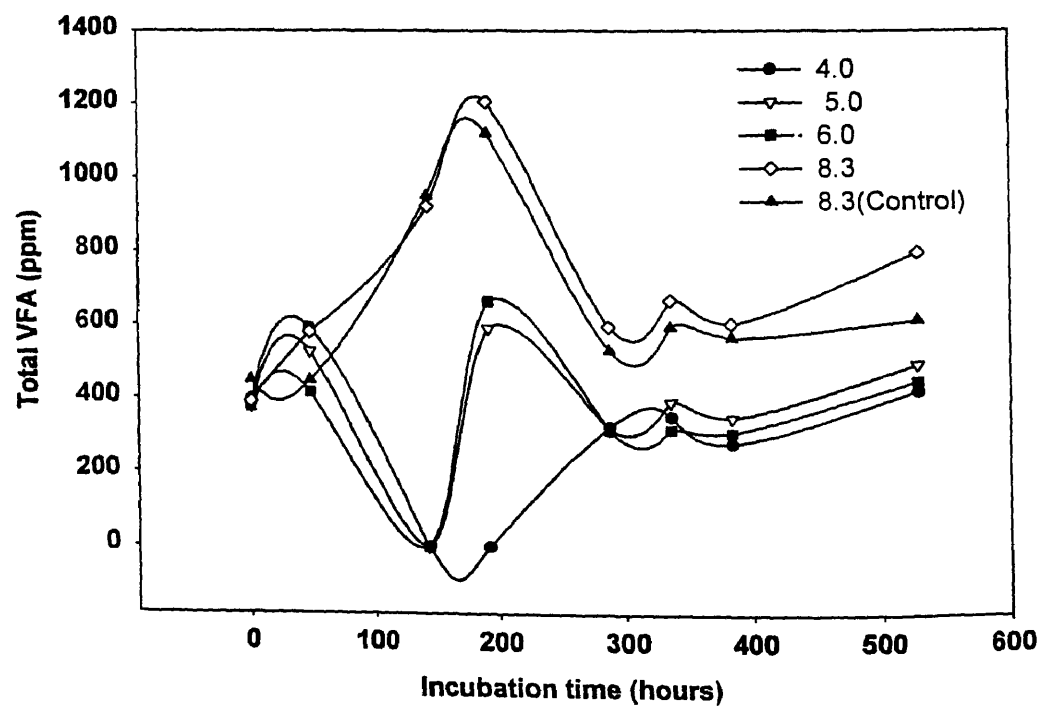


Figure 98. Effect of pH on change in VFA production on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

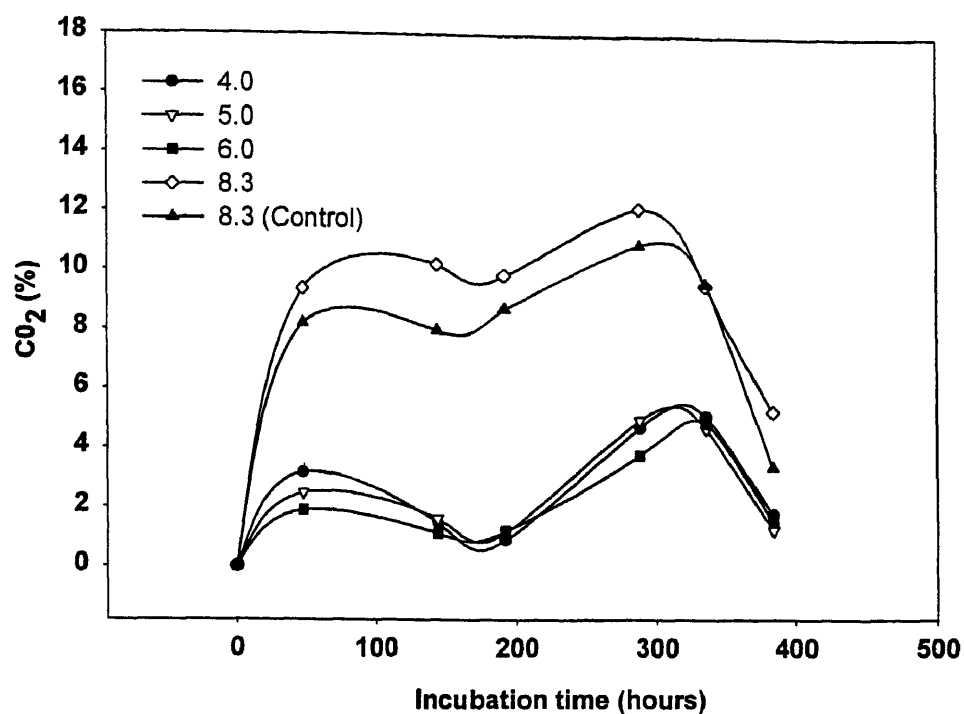


Figure 99. Effect of pH on change in CO₂ production on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

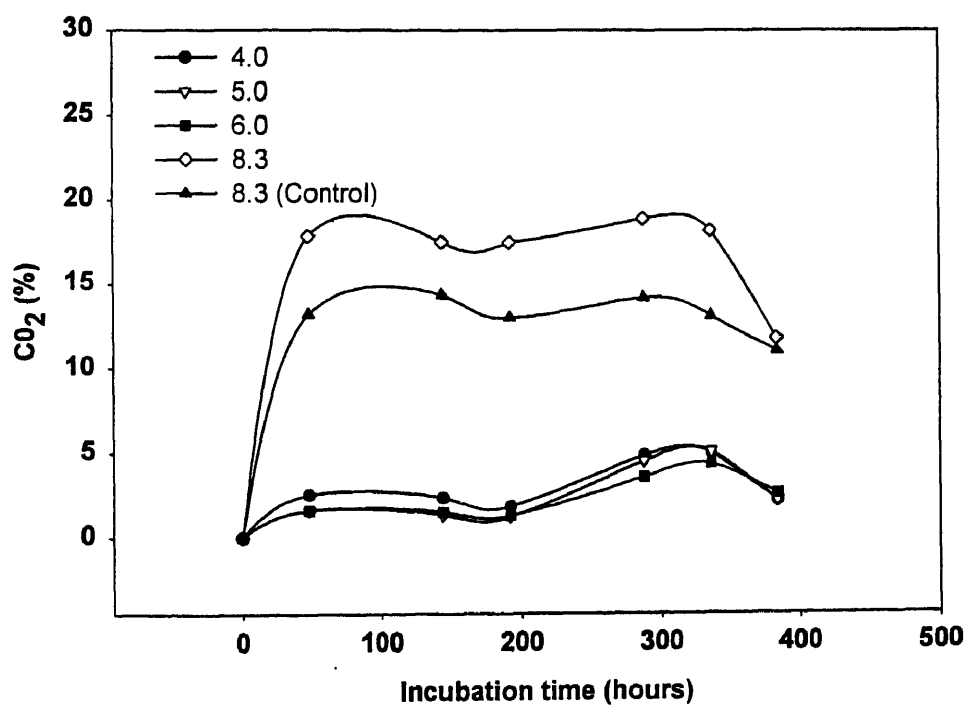


Figure 100. Effect of pH on change in CO₂ production on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

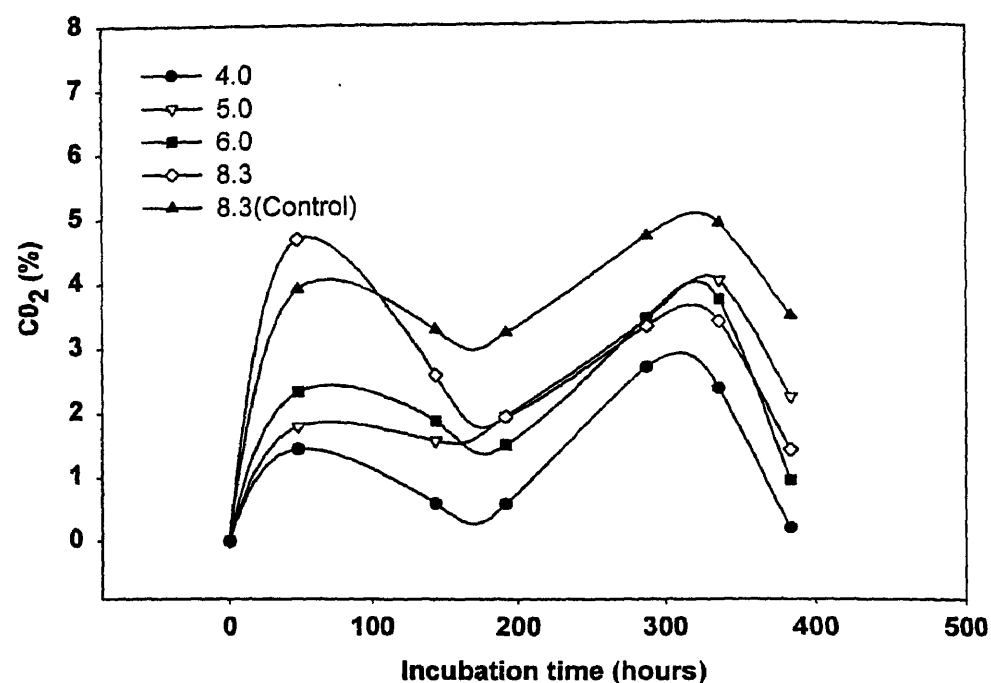


Figure 101. Effect of pH on change in CO₂ production on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

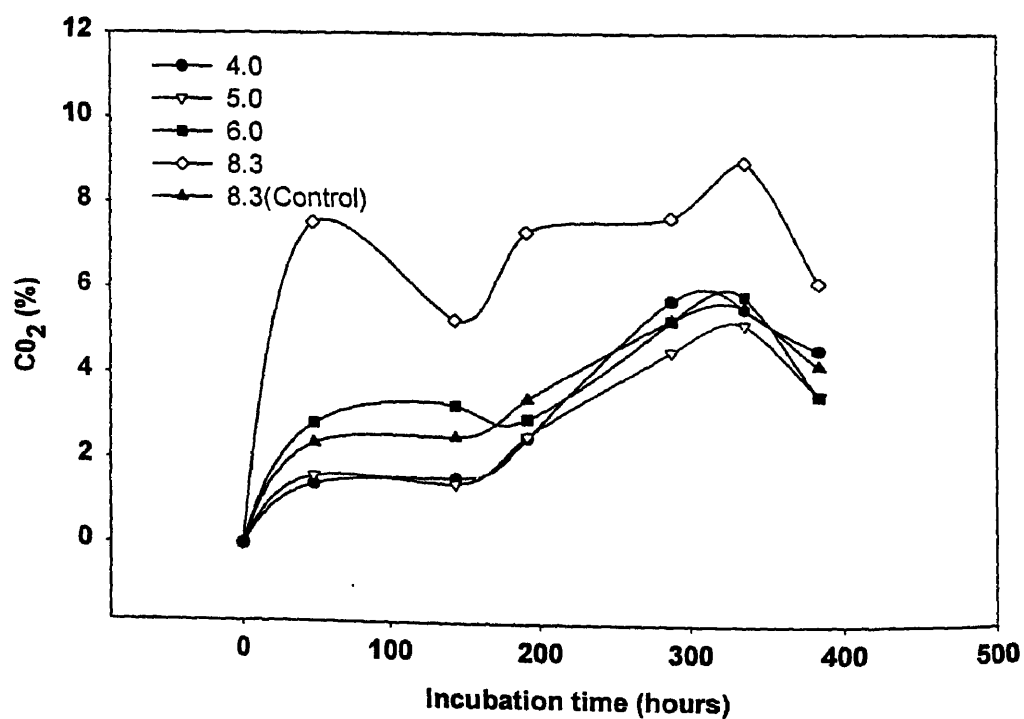


Figure 102. Effect of pH on change in CO₂ production on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

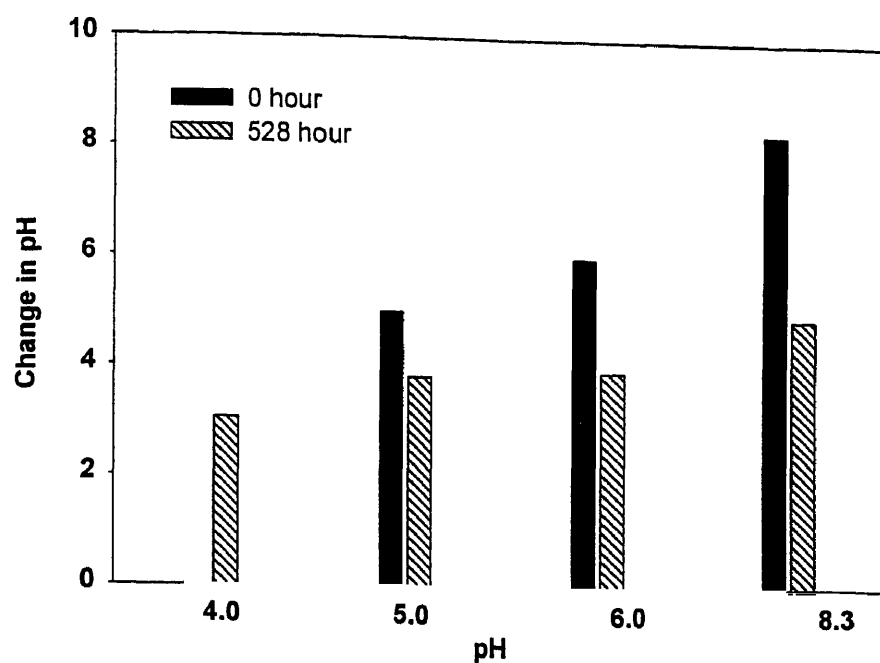


Figure 103. Effect of pH on change in pH on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

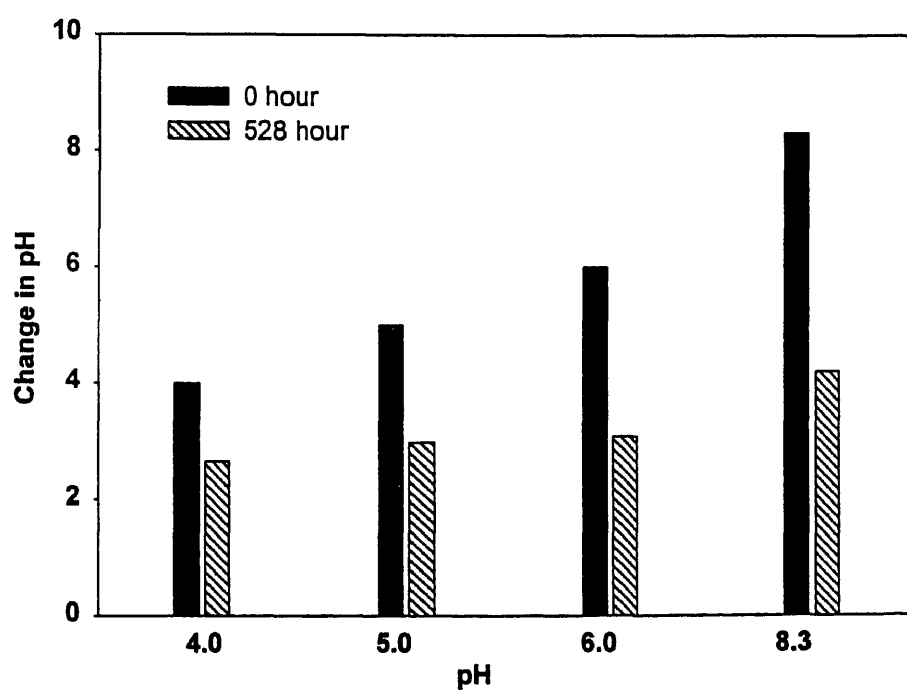


Figure 104. Effect of pH on change in pH on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

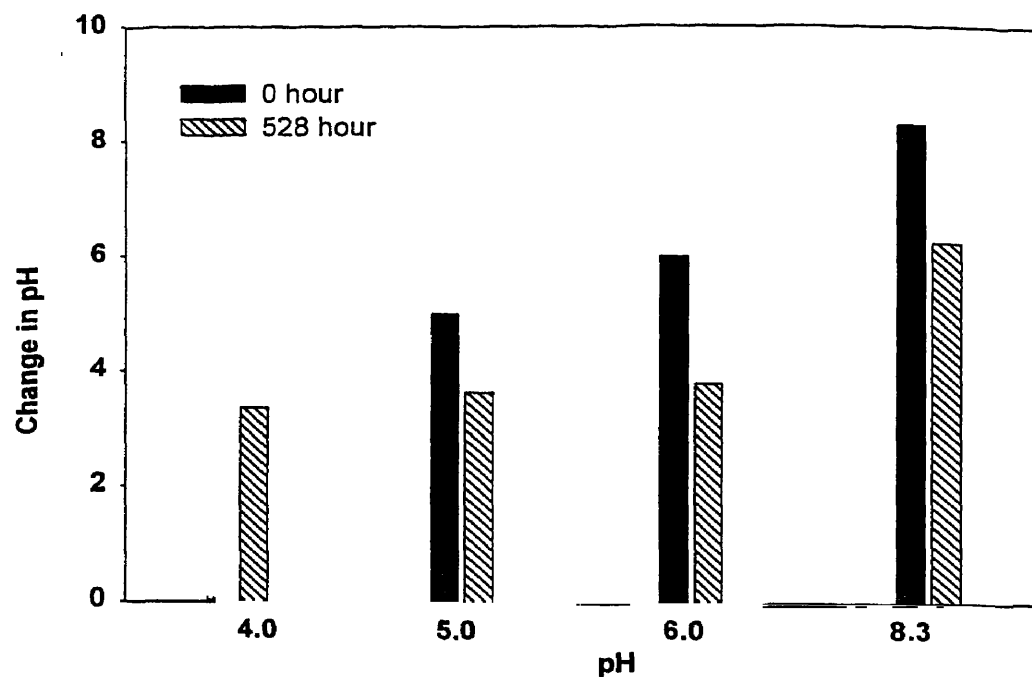


Figure 105. Effect of pH on change in pH on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

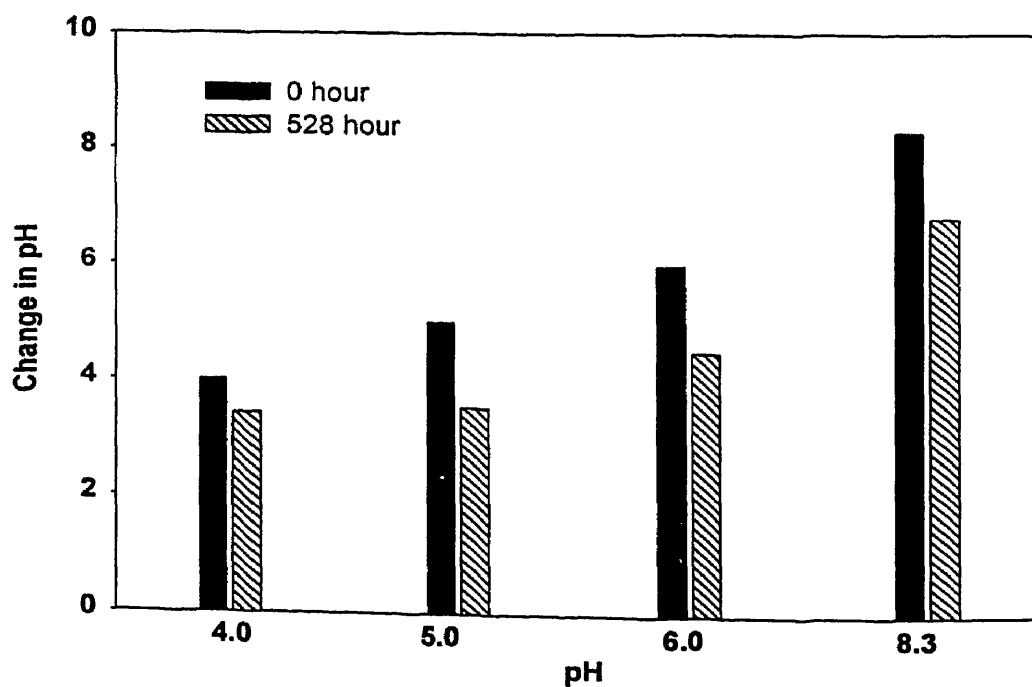


Figure 106. Effect of pH on change in pH on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

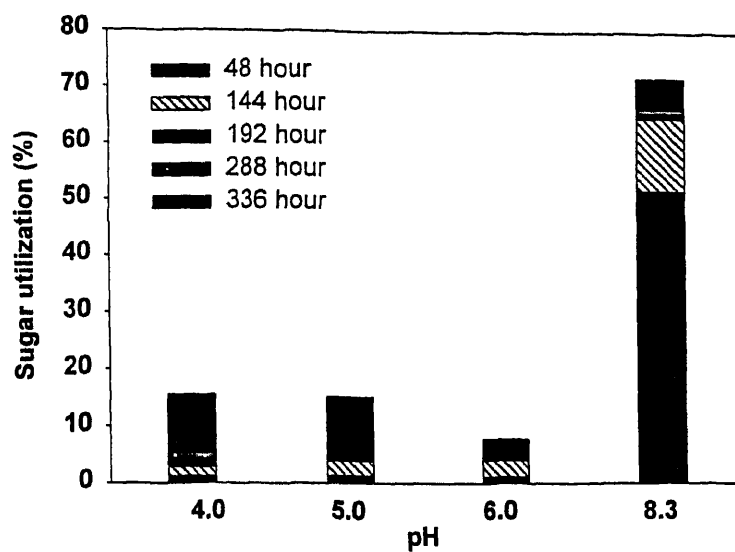


Figure 107. Effect of pH on total sugars utilization on glucose (single strength) as carbon source during growth of S-2 consortium at 90 °C

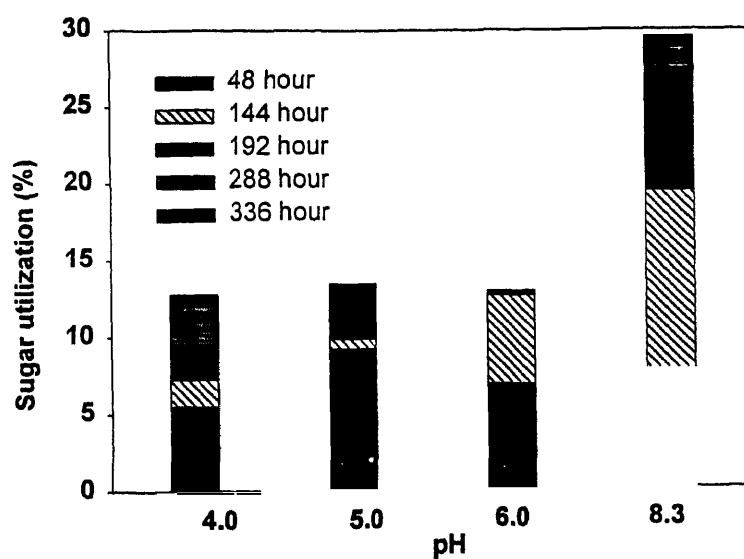


Figure 108. Effect of pH on total sugars utilization on glucose (double strength) as carbon source during growth of S-2 consortium at 90 °C

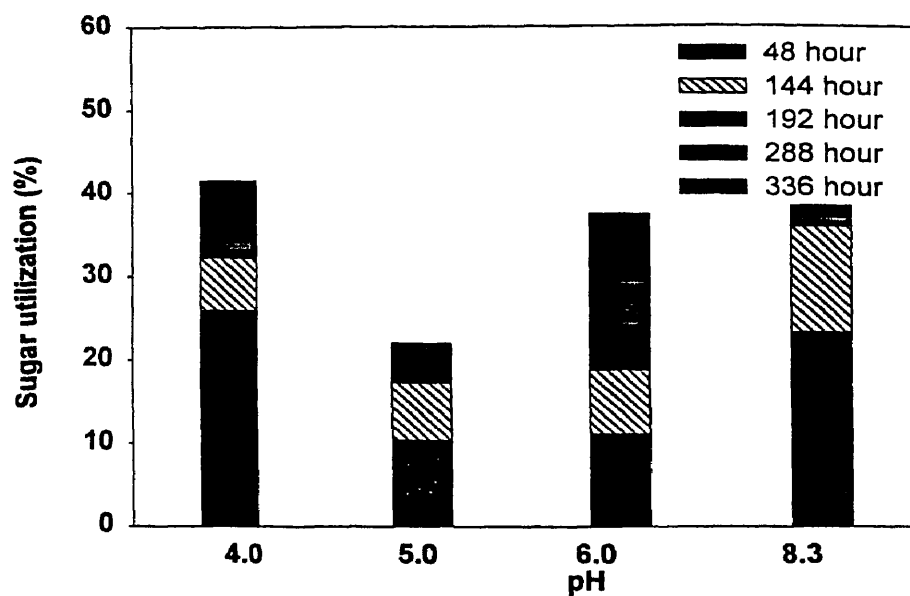


Figure 109. Effect of pH on total sugars utilization on molasses (single strength) as carbon source during growth of S-2 consortium at 90 °C

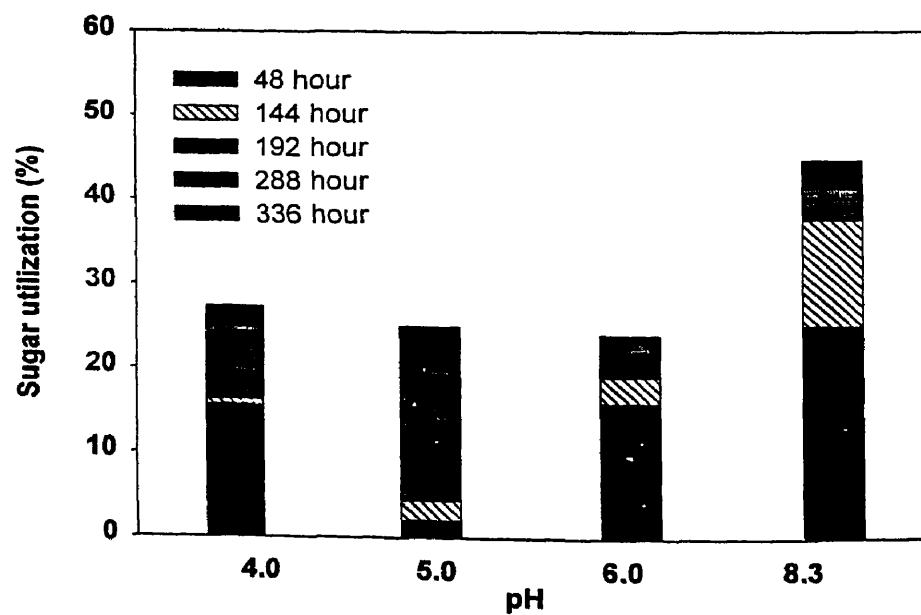


Figure 110. Effect of pH on total sugars utilization on molasses (double strength) as carbon source during growth of S-2 consortium at 90 °C

Pathogenicity test of selected anaerobic consortium J1 and S2

The consortium J-1 and S-2 were then analysed for pathogenicity at the All India Institute of Medical Sciences (AIIMS), New Delhi. The results indicated that the consortium J-1 was pathogenic to mice while the consortium S-2 was not pathogenic in nature. These results thus indicated that consortium S-2 could be used for all further studies in the core flood trial and final EOR process (Table 8).

Table 8 Animal pathogenicity test of selected anaerobic consortium J1 and S2

S. No.	Culture No.	Guinea pig pathogenicity test			Mouse pathogenicity test	
		IMI (RT leg)	IMI (left leg)	Results	IPI	Results
1.	J-1	26.5.2000 died after 5 days		Pathogenic for guinea pig	26.5.2000 1 mouse died after 1½ month after receiving dose of sample (1.5 ml)	Pathogenic for mice
2.	J-2	26.5.2000 to 30.6.2000	1.7.2000 to 26.7.2000	Alive & healthy	26.5.2000 1 mouse died after 10 days	Pathogenic for mice
3.	S-2	26.5.2000 to 30.6.2000	1.7.2000 to 26.7.2000	Alive & healthy	26.5.2000 to 26.7.2000	Alive & healthy
4.	Control	26.5.2000 to 30.6.2000	1.7.2000 to 26.7.2000	Alive & healthy	26.5.2000 to 26.7.2000	Alive & healthy
5.	<i>Clostridium tetani</i> Mice		+ve control -ve control	Died on 15.6.2000 Alive and healthy		

Conclusion: Samples J-1 and J-2 are not suitable for use, as they are pathogenic for mice

Preservation and maintenance of anaerobic, thermophilic consortia

A 50% (v/v) glycerol solution is prepared (Appendix) anaerobically. A 10% glycerol (v/v) solution was added anaerobically with the help of air-tight sterilized syringe to the liquid culture. The culture temperature is gradually reduced to freezing and then stored at -40°C in deep freezer. A routine check-up of the culture viability needs to be performed once or twice a year from a duplicate culture vial every time.

Core flood trial with developed S-2 consortium at 90°C

The core flood study with the selected S-2 consortium was under taken at IRS, Ahmedabad to test the feasibility of use of S-2 consortium for EOR process in the field. The experimental details and results (as provided by IRS, Ahmedabad) are presented in Tables 9.

During 21 days of incubation of oil containing core at 90°C, an 8.93% incremental recovery of oil by MEOR over water flood and 21.0% residual oil recovery was achieved.

Table 9 Experimental details and results on core flood study

<i>Experimental details</i>	
Incubation period	21 days
Core plug used	Barea
Length	19.88 cm
Diameter	3.836 cm
Area	11.523 cm ²
Porosity	19.0%
Kair	148 md
Pore volume	43 CC
Oil used	Jotana # 57
Water	With salinity of 20 g of KCl in one litre
Temperature	90°C
Rate of injection	20 CC/hour
<i>Test results</i>	
Soi	65.12% of PV
SOR by water flood followed by MEOR flood	27.9% of PV
SORW after incubation and production	22.1% of PV
Oil recovery by water flood	57.14%
Total recovery	66.07%
Incremental recovery by MEOR over water flood	8.93%
% recovery of residual oil	21.0
% of CO ₂ in the produced gas	1.8

Development of aerobic consortia for biosurfactant production

Samples (six) collected from Manikaran were grown on basal mineral medium as well as Luria broth with 1% (v/v) crude oil at 55 °C and 70°C respectively. Very less growth was observed at 70°C on basal mineral medium with 1% (v/v) crude oil or on Luria broth with 1% (v/v) crude oil except for one sample which gave good growth at both 55°C and 70°C on both the medium (Figure 111). This sample was then selected for further enrichment for the selection of bacterial consortium capable of producing thermostable biosurfactant.

Subsequently four samples were also collected from oil well in the Mehsana region and the development of the aerobic consortia was initiated on basal mineral medium as well as Luria broth with 1% (v/v) crude oil at 55°C and 70°C respectively. Again no growth was observed on basal mineral medium with 1% (v/v) crude oil as the sole source of carbon. Good growth was observed on Luria broth with 1% (v/v) crude oil in all the four samples from Mehsana region and were then enriched at both 55°C and 70°C respectively. After repeated enrichment the biosurfactant activity was measured as emulsifying activity as described in details in the method section.

Among the four samples from the oil wells of Mehsana region the sample from well-91 was the best with thermostable biosurfactant activity followed by the sample from Manikaran (Figures 111 to 120). Maximum activity in Ah-91 was observed after 96 hours of growth with maximum thermostability at 80°C with 30% loss of activity at 90°C (Figures 115 & 116). Sample Mn-4 showed the next highest activity but was only 60% of that observed with Ah-91 (Figures 111 & 112). The above consortia Mn-4 and Ah-91 were thus selected for the isolation and screening of the various isolates comprising this consortia. Consortium Mn-4 was plated on Luria agar plate around and isolated colonies so obtained were screened for production of thermostable biosurfactant activity.

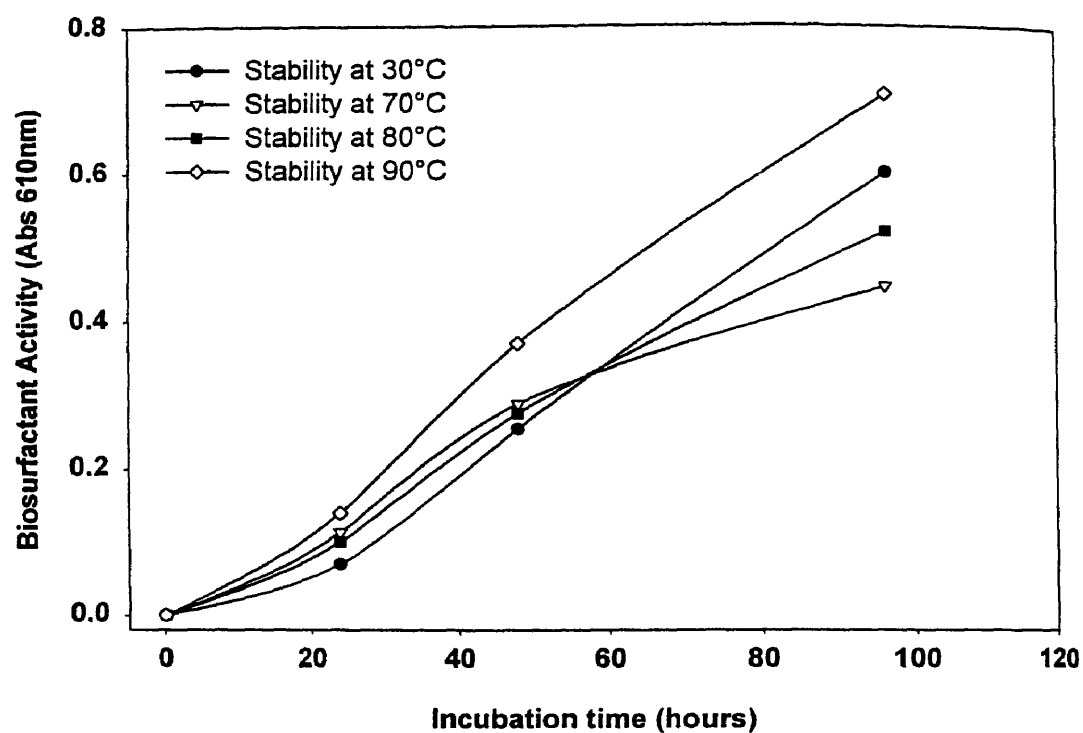


Figure 111. Biosurfactant production by Mn-4 grown at 55°C

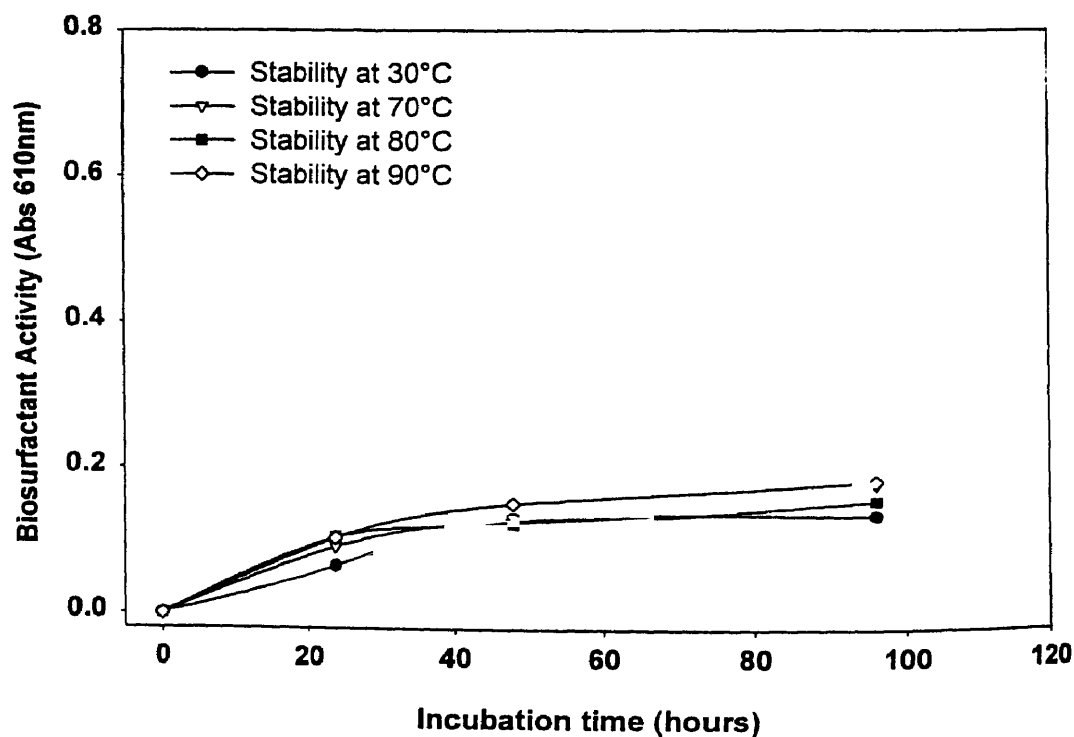


Figure 112. Biosurfactant production by Mn-4 grown at 70°C

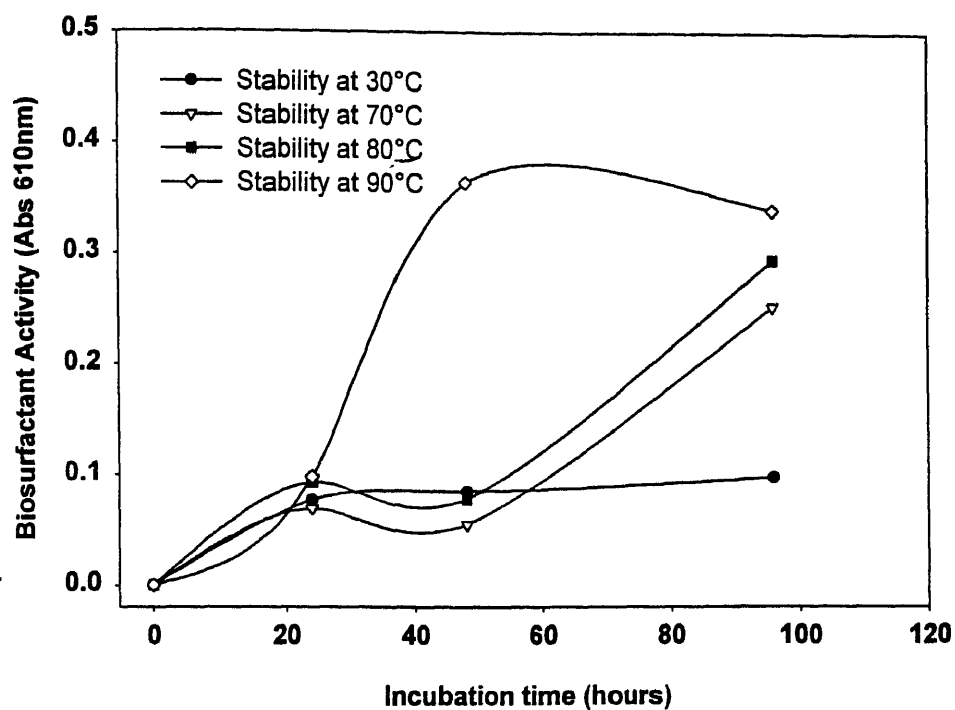


Figure 113. Biosurfactant production by Ah-32 grown at 55°C

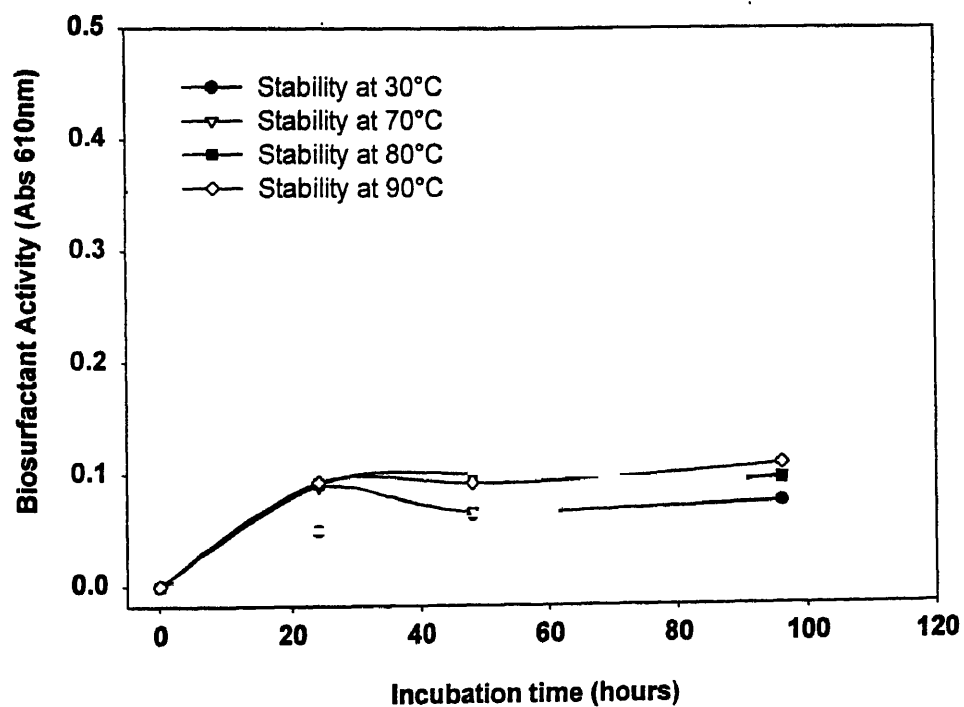


Figure 114. Biosurfactant production Ah-32 grown at 70°C

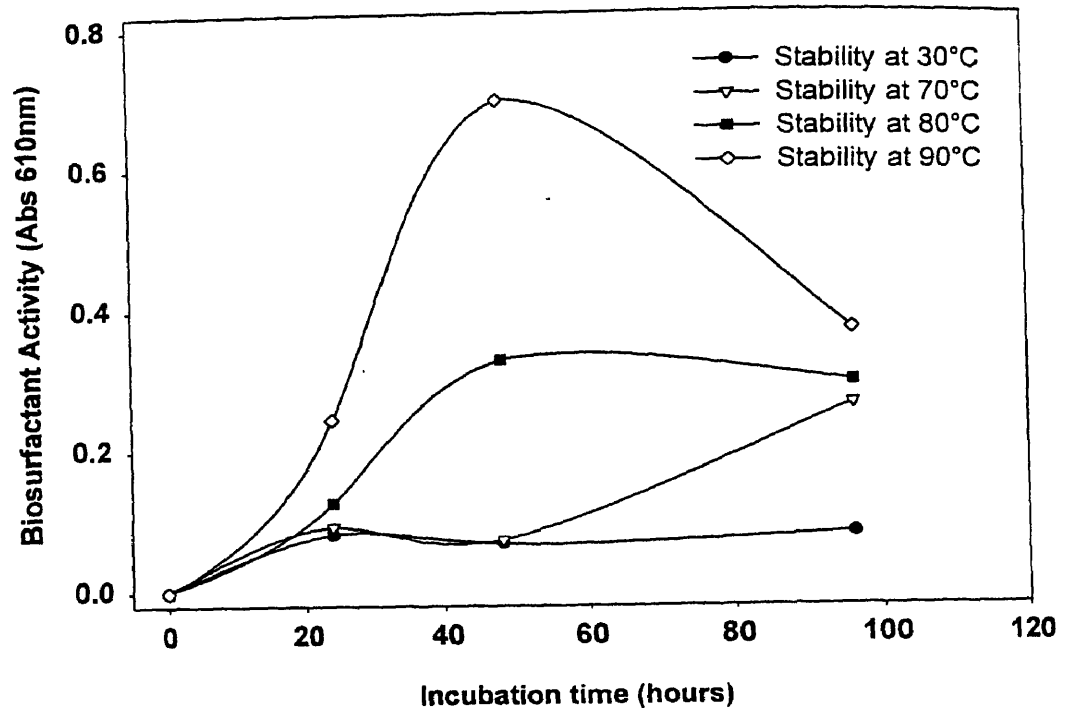


Figure 115. Biosurfactant production by Ah-91 grown at 55°C

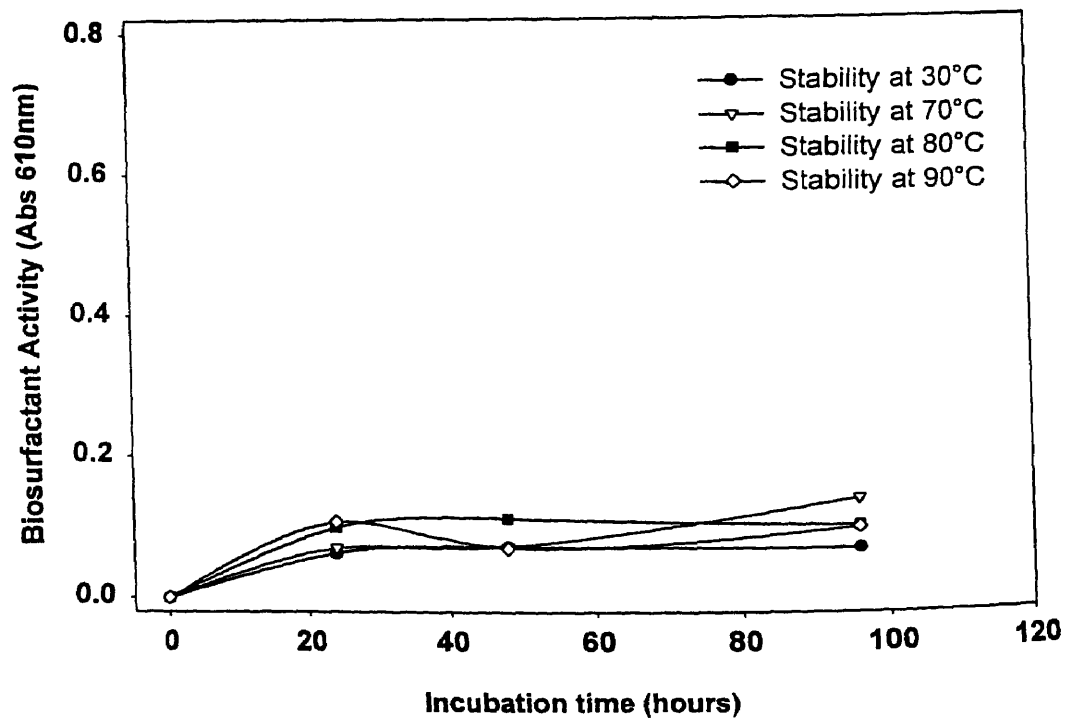


Figure 116. Biosurfactant production by Ah-91 grown at 70°C

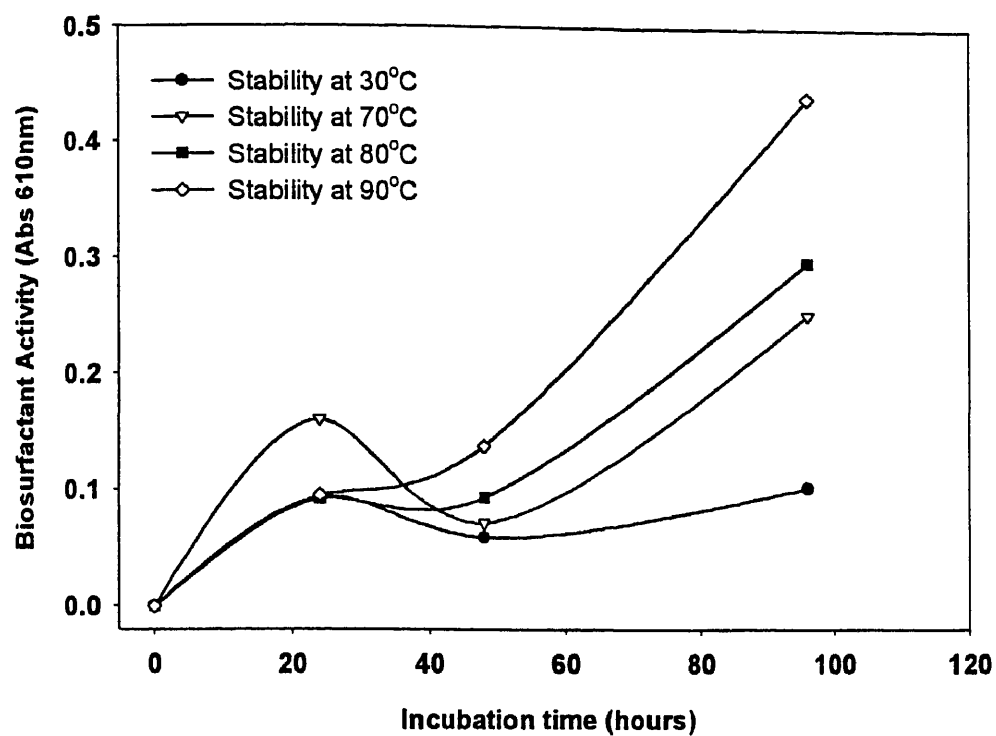


Figure 117. Biosurfactant production by Ah-102 grown at 55°C

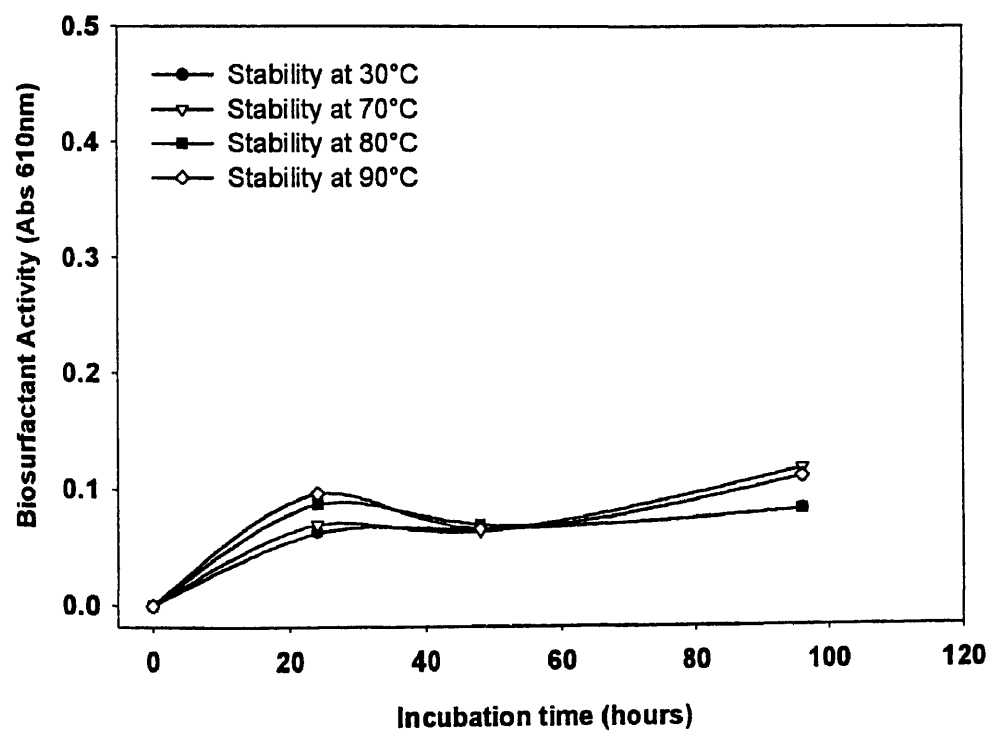


Figure 118. Biosurfactant production by Ah-102 grown at 70°C

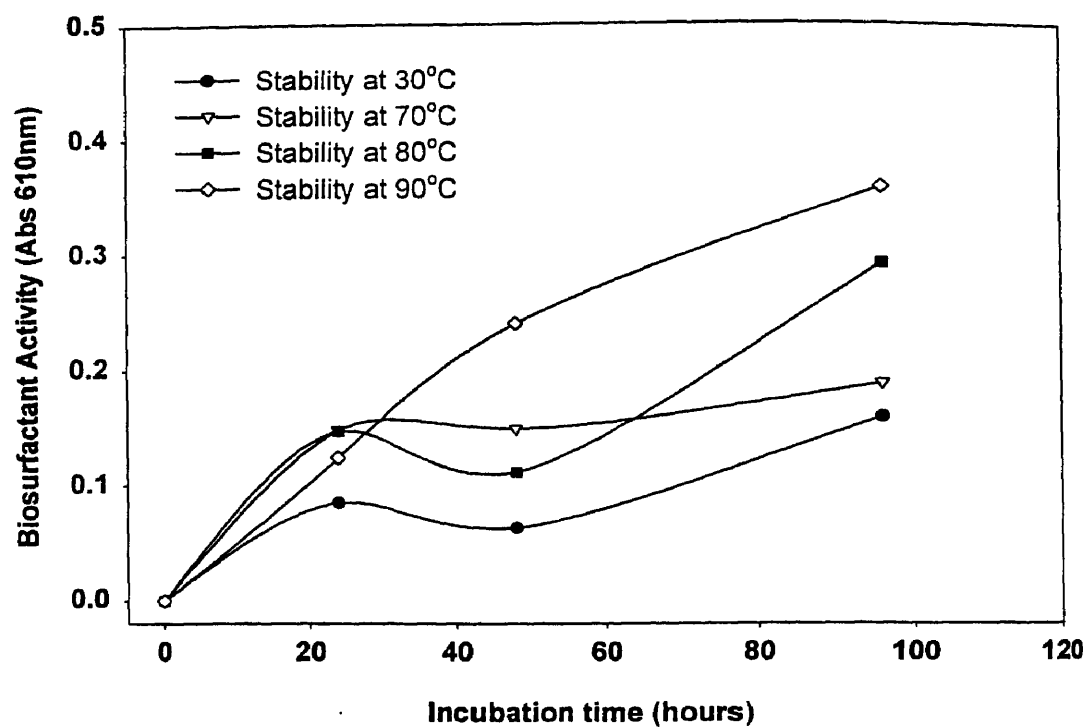


Figure 119. Biosurfactant production by Ah-109 grown at 55°C

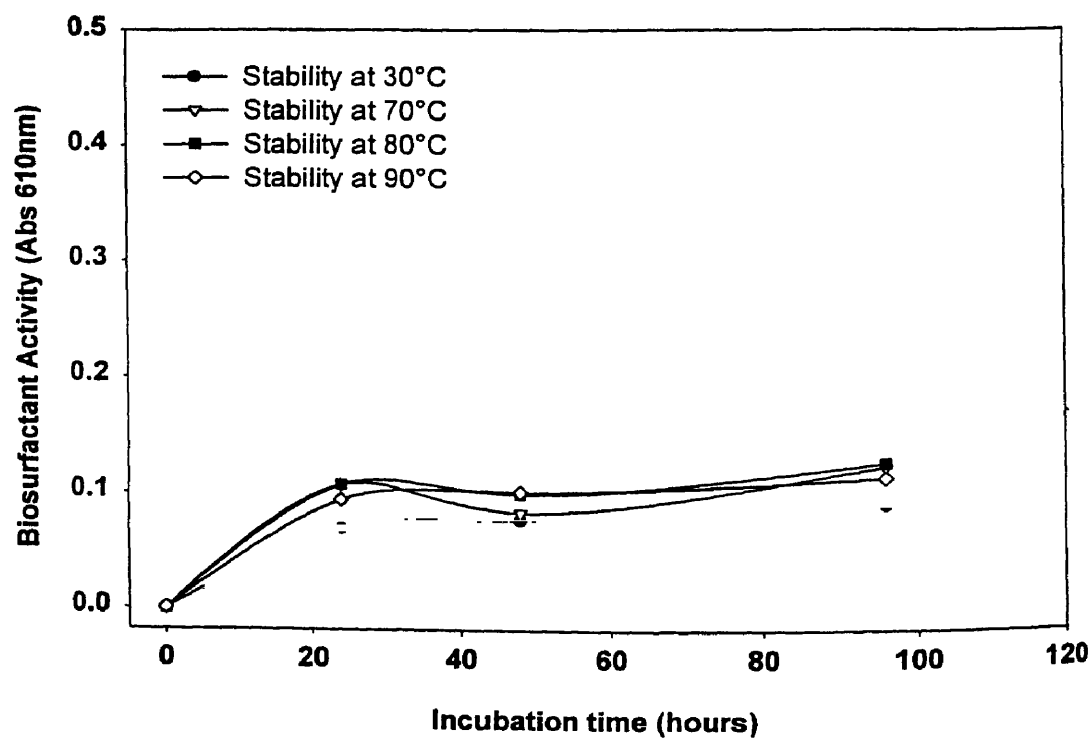


Figure 120. Biosurfactant production by Ah-109 grown at 70°C

Among the six samples collected from Manikaran only one sample Mn-4 showed good growth at both 55°C and 70°C while among the four samples from the Mehsana region, sample Ah-91 was the best, showing production of biosurfactant with thermal stability at both 80°C and 90°C (Figure 121). Further the growth and biosurfactant activity of the consortium Mn-4 and Ah-91 was very good at 55°C but not so at 70°C.

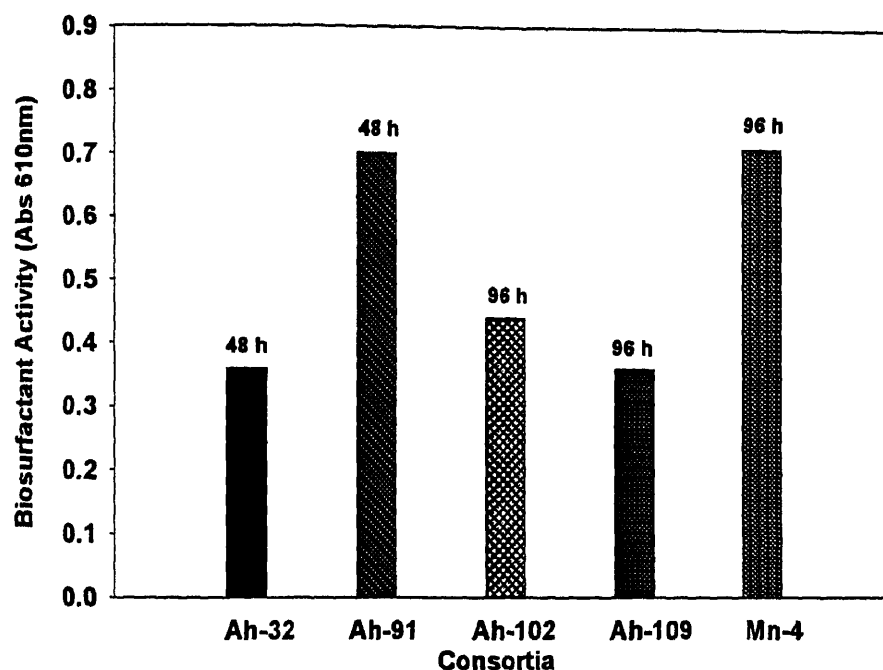


Figure 121. Biosurfactant production by consortia from Mehsana and Manikaran

Consortia Mn-4 was plated on Luria-phytagel plates and 261 isolated colonies were selected for screening for the production of thermostable biosurfactant activity. Analysis of the thermostable biosurfactant activity showed that there was considerable variability among the different isolates with respect to the level and the time at which the maximum thermostable activity was observed during growth of these isolates (Figures 122 to 128). Of the 261 isolates from the Manikaran consortium (Mn-4), 23 isolates showing the maximum thermostable biosurfactant activity were selected for further screening (Figure 129). The best five isolates Mn-131, Mn-175, Mn-182, Mn-191, and Mn-212 were selected and studied for their tolerance to 90°C at various concentration of NaCl ranging from 1-4% and biosurfactant activity with different concentrations of oil.

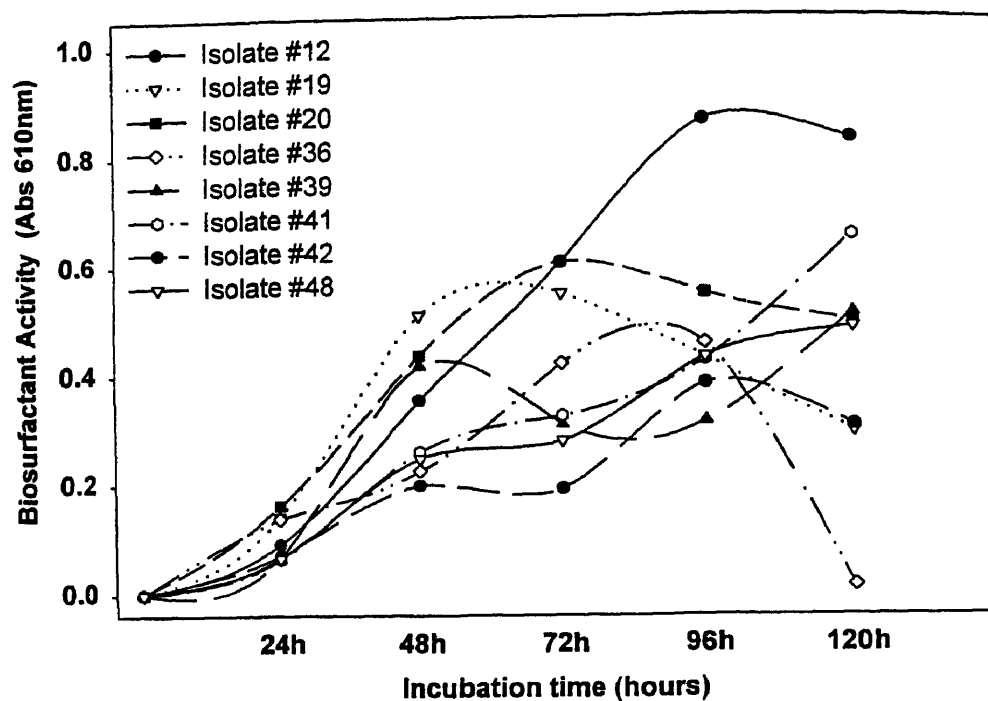


Figure 122. Biosurfactant production by Manikaran isolates #Mn12-Mn48

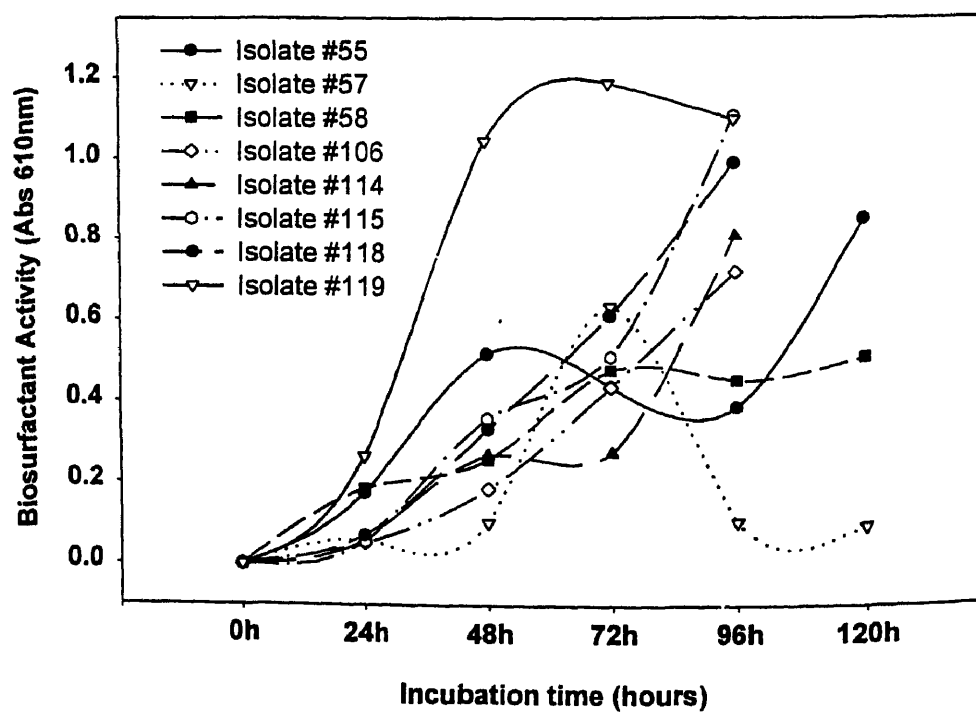


Figure 123. Biosurfactant production by Manikaran isolates #Mn 55-119

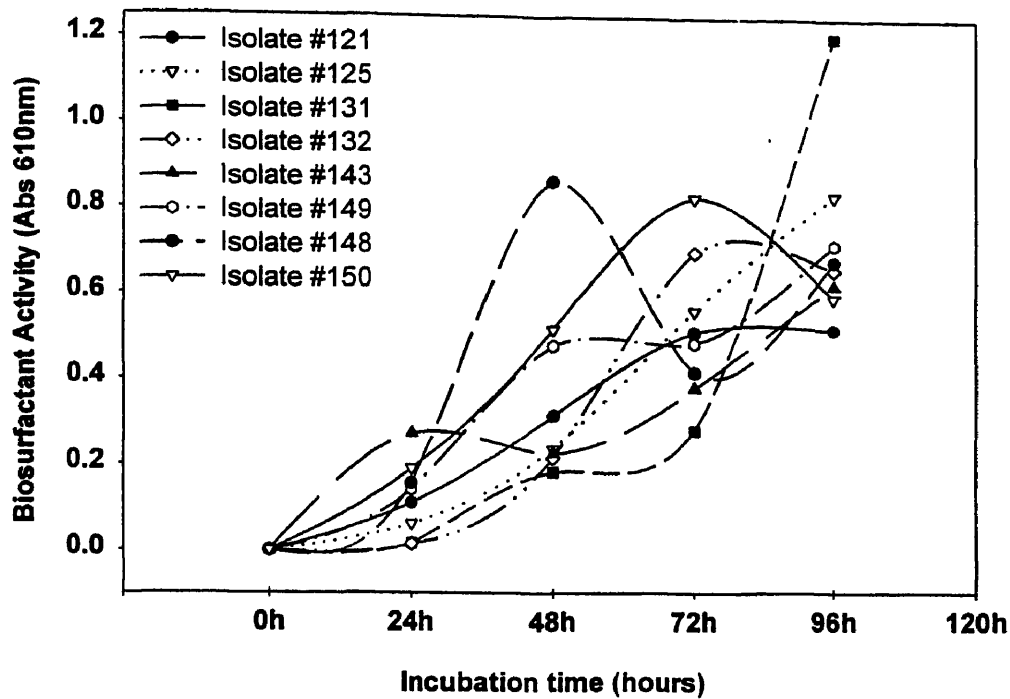


Figure 124. Biosurfactant production by Manikaran isolates # Mn121-150

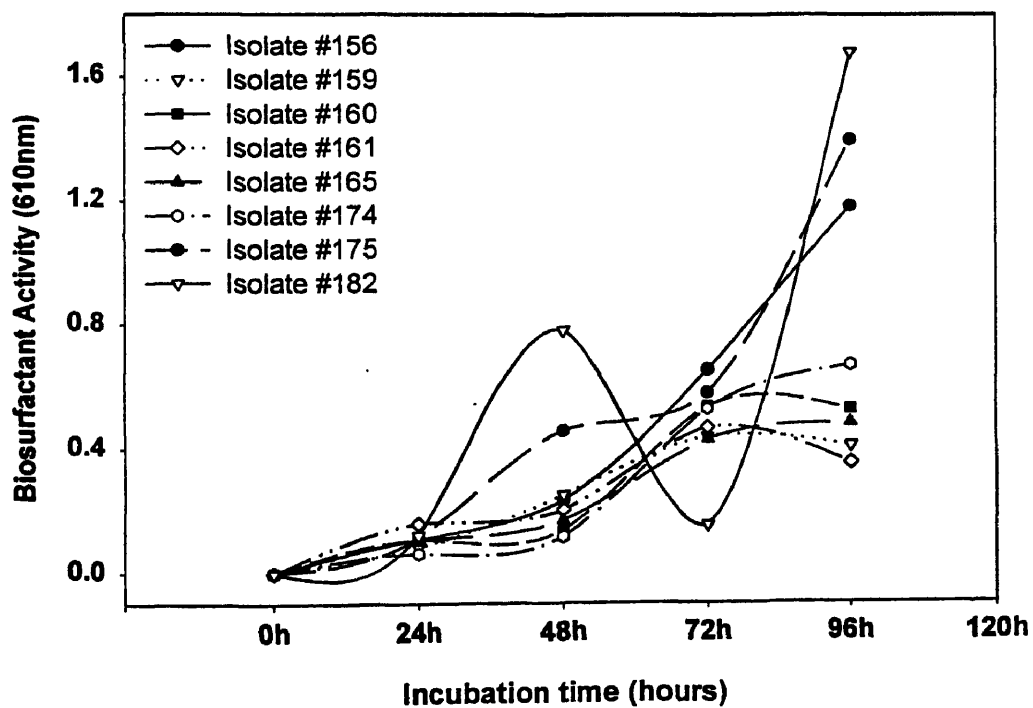


Figure 125. Biosurfactant production by Manikaran isolates #Mn156-182

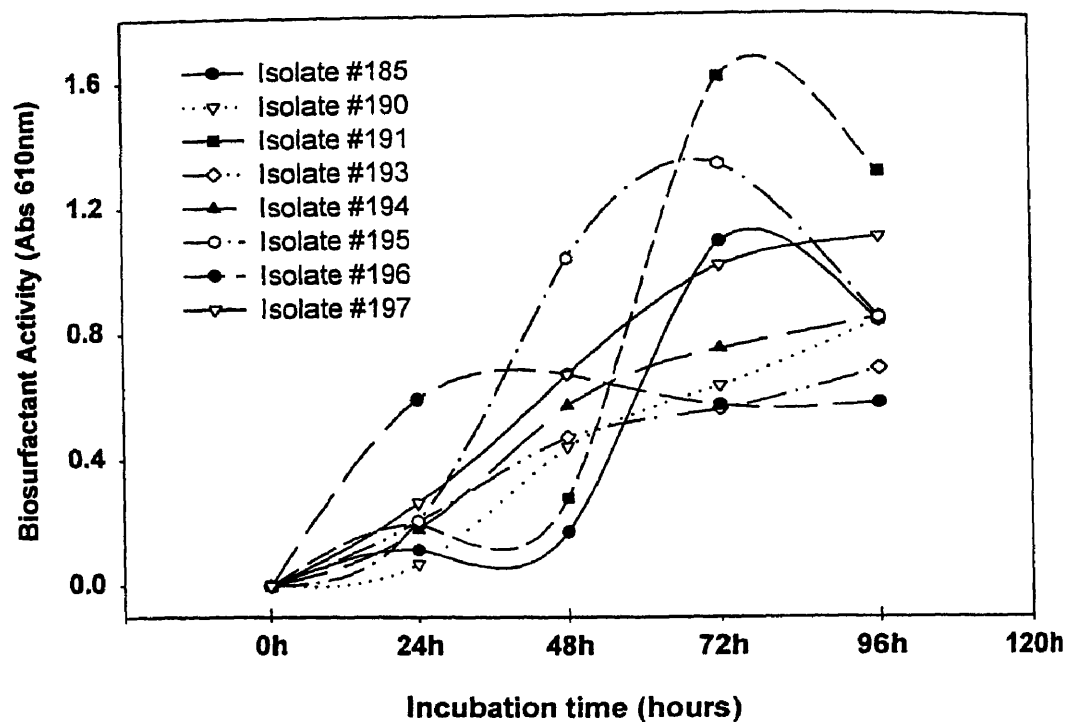


Figure 126. Biosurfactant production by Manikaran isolates #Mn185-197

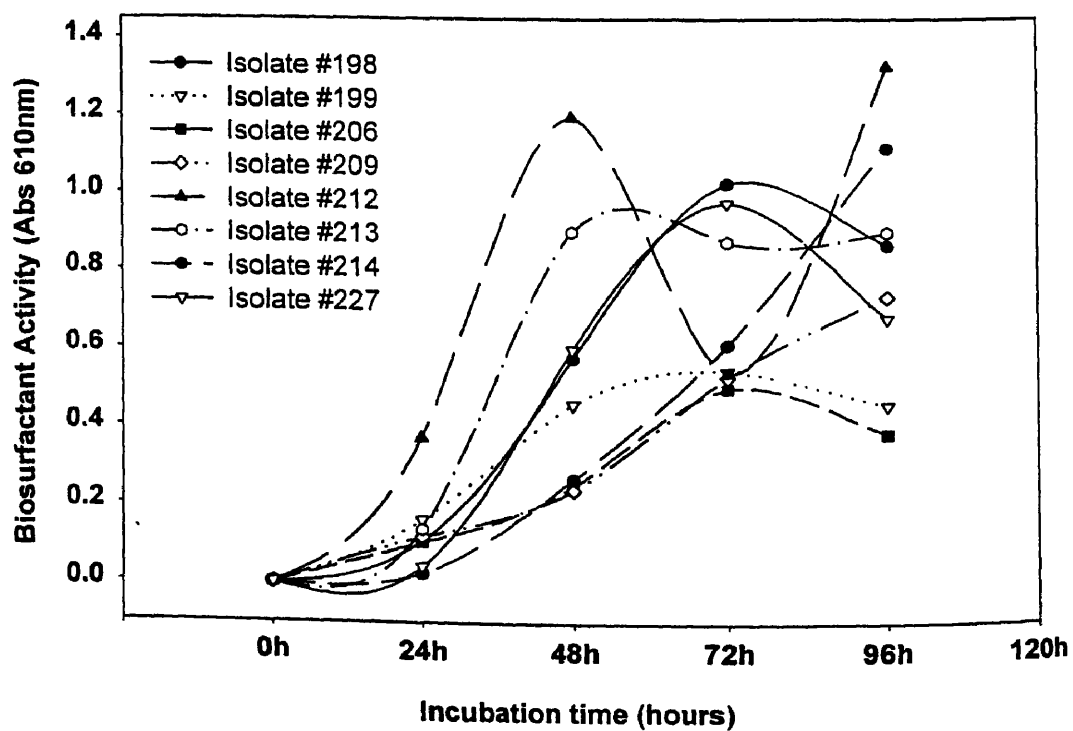


Figure 127. Biosurfactant production by Manikaran isolates # Mn198-227

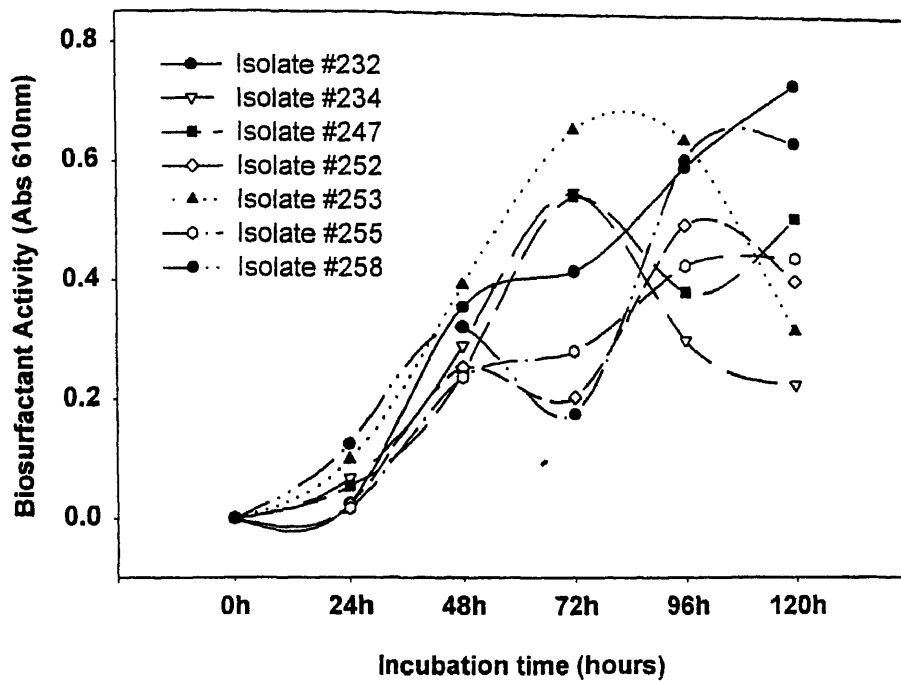


Figure 128. Biosurfactant production by ManiKaran isolates # Mn232-258

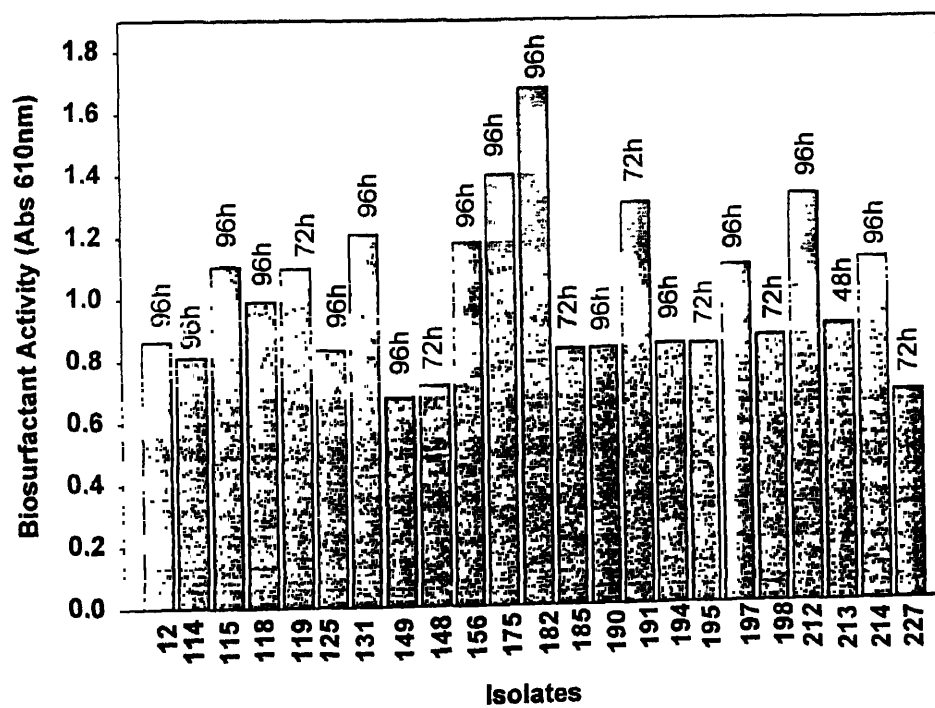


Figure 129. Biosurfactant production by selected isolates of Manikaran consortia Mn-4

Consortium Ah-91 was also plated on Luria-phytagel plates and 116 isolated colonies were selected for screening for the thermostable biosurfactant activity over 120 hours at 24 hours intervals. Analysis revealed that as with Mn-4, there was also considerable variability among the various isolates from Ah-91 with respect to the level and the time at which the maximum thermostable activity was observed during growth of these isolates. Among these 116 isolates, the best 13 with maximum thermostable biosurfactant activity were selected for further studies with respect to their tolerance to NaCl concentration (1-4%) and also different concentration of oil. An interesting feature of all the isolates screened from consortium Ah-91 was that the maximum thermotolerant biosurfactant activity was observed within 48 hours of the growth of the isolate unlike that with Mn-4 where the maximum thermotolerant biosurfactant activity was observed only after 72 hours or 96 hours of growth.

Five samples from Konkan region were screened for their biosurfactant production activity at 55°C (Figure 130). Among the five consortium developed from these six samples, consortia Kon-1 was the best with maximum biosurfactant activity at 72 hours. Consortia Kon-1 was then plated on phytagel and 72 isolates so obtained were then screened for biosurfactant activity. All the 72 isolates showed maximum biosurfactant activity after 96 hours of growth with stability of the biosurfactant at 90°C. Among these 72 isolates, the best 12 were selected for further studies (Figures 131 to 142).

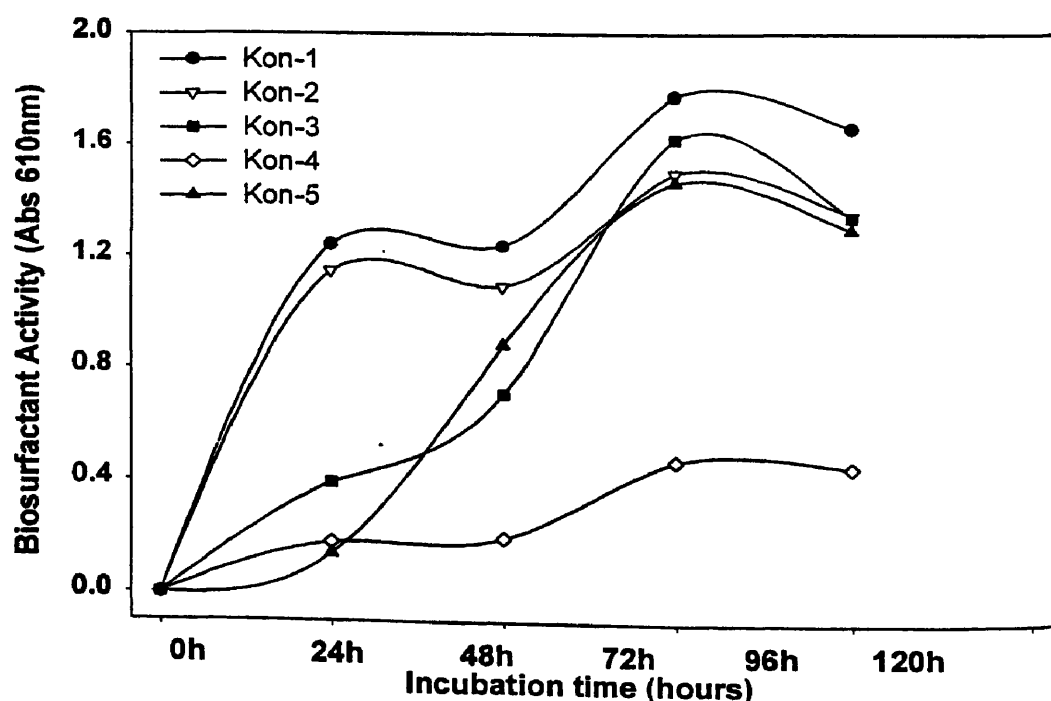


Figure 130. Production of biosurfactant by Konkan consortia

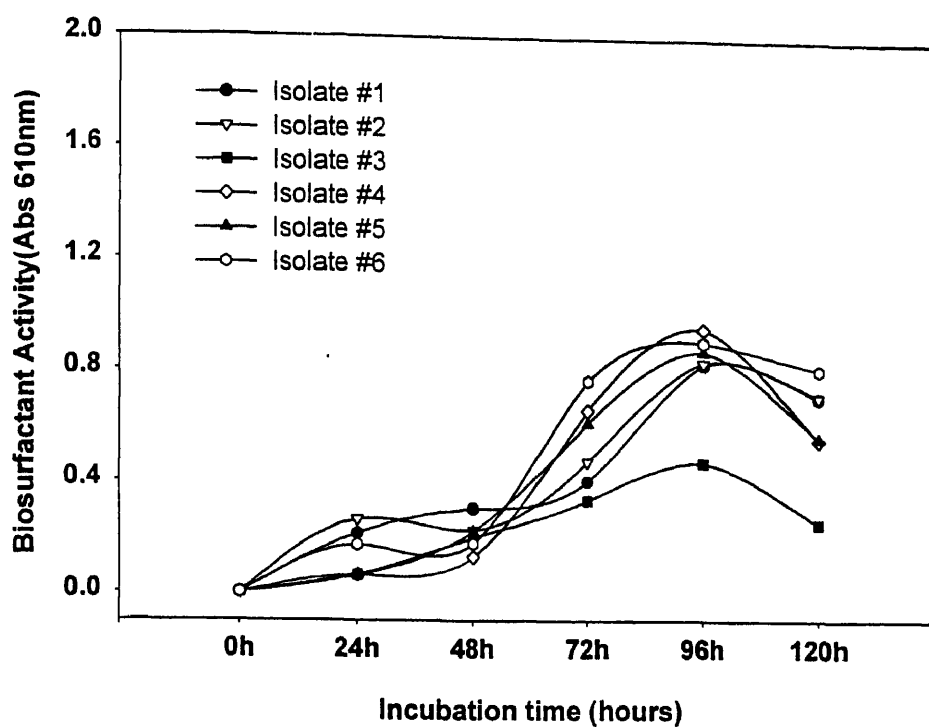


Figure 131. Biosurfactant production by Konkan isolates # 1-6

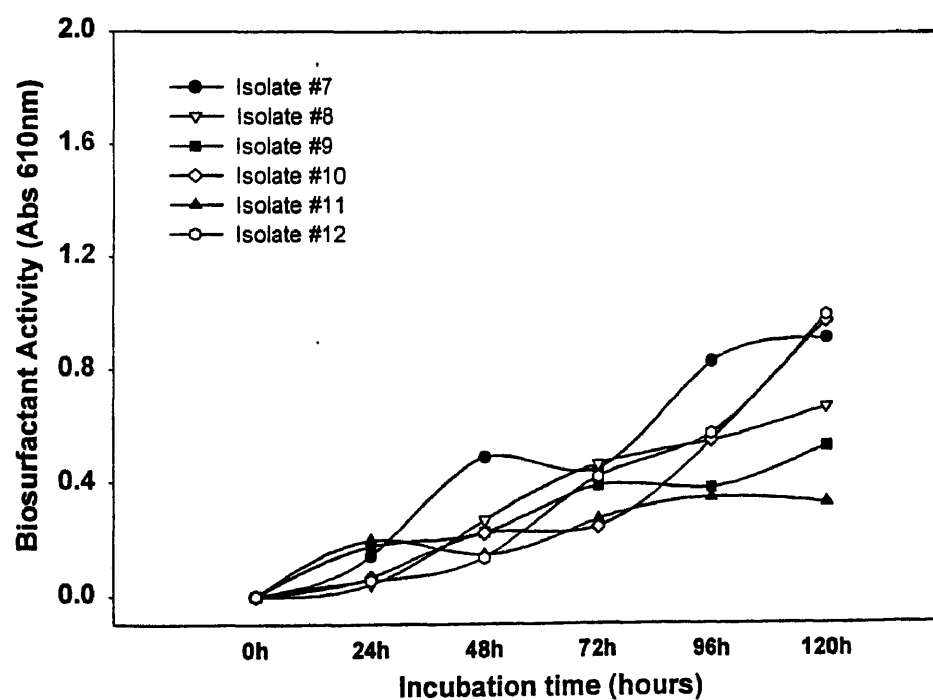


Figure 132. Biosurfactant production by Konkan isolates # 7-12

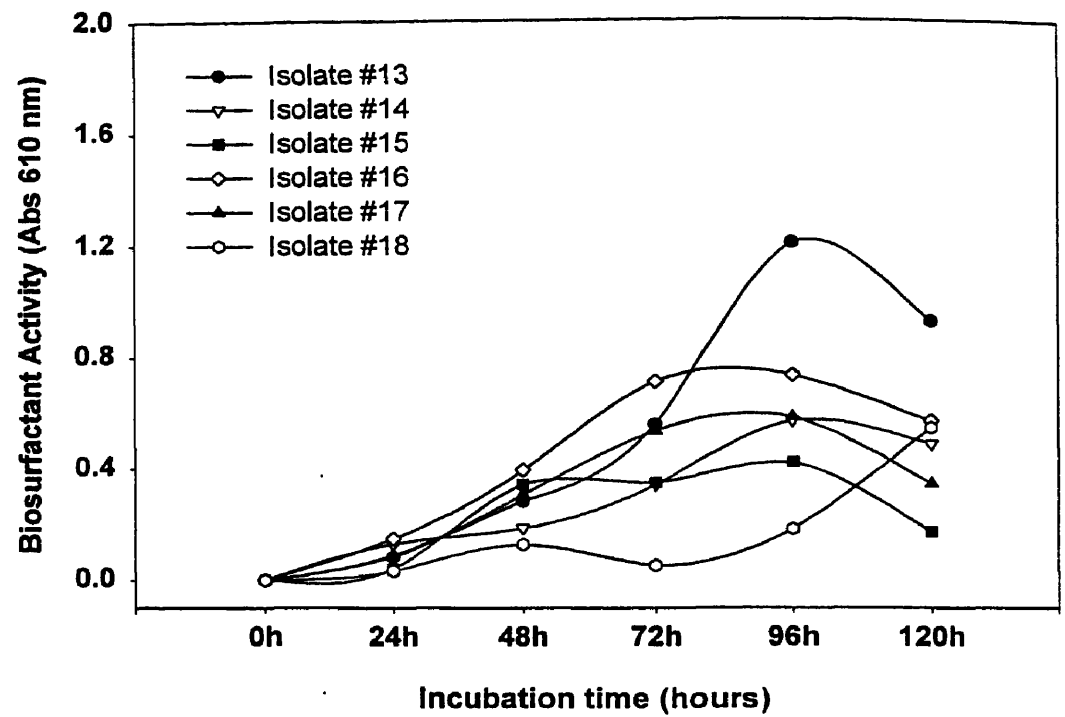


Figure 133. Biosurfactant production by Konkan isolates # 13-18

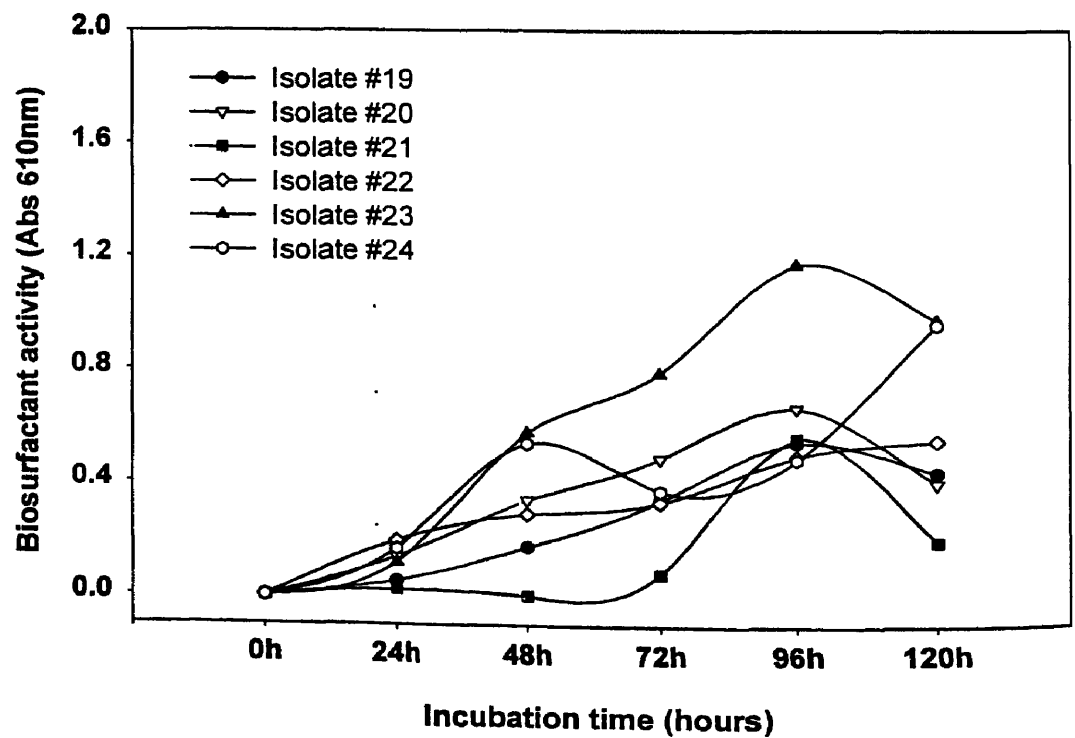


Figure 134. Biosurfactant production by Konkan isolates # 19-24

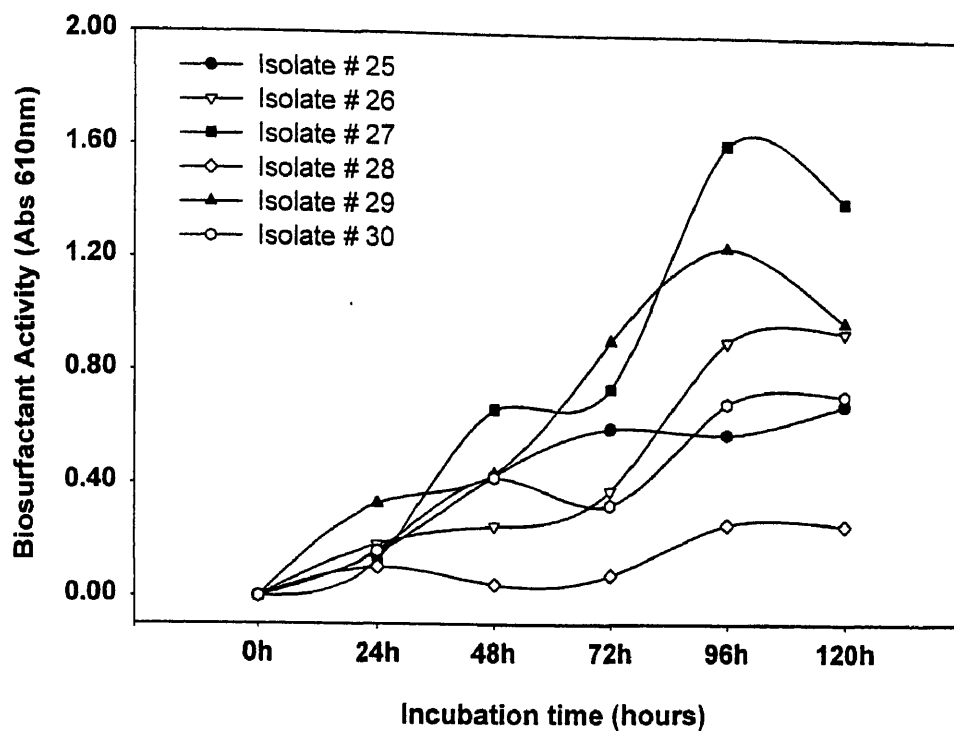


Figure 135. Biosurfactant production by Konkan isolates # 25-72

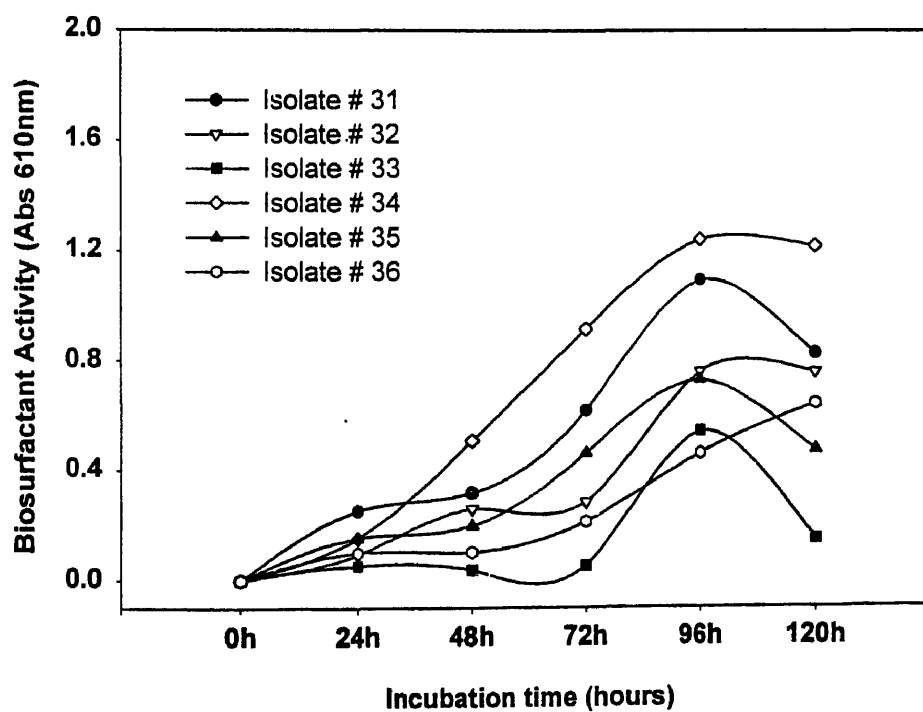


Figure 136. Biosurfactant production by Konkan isolates # 31-36

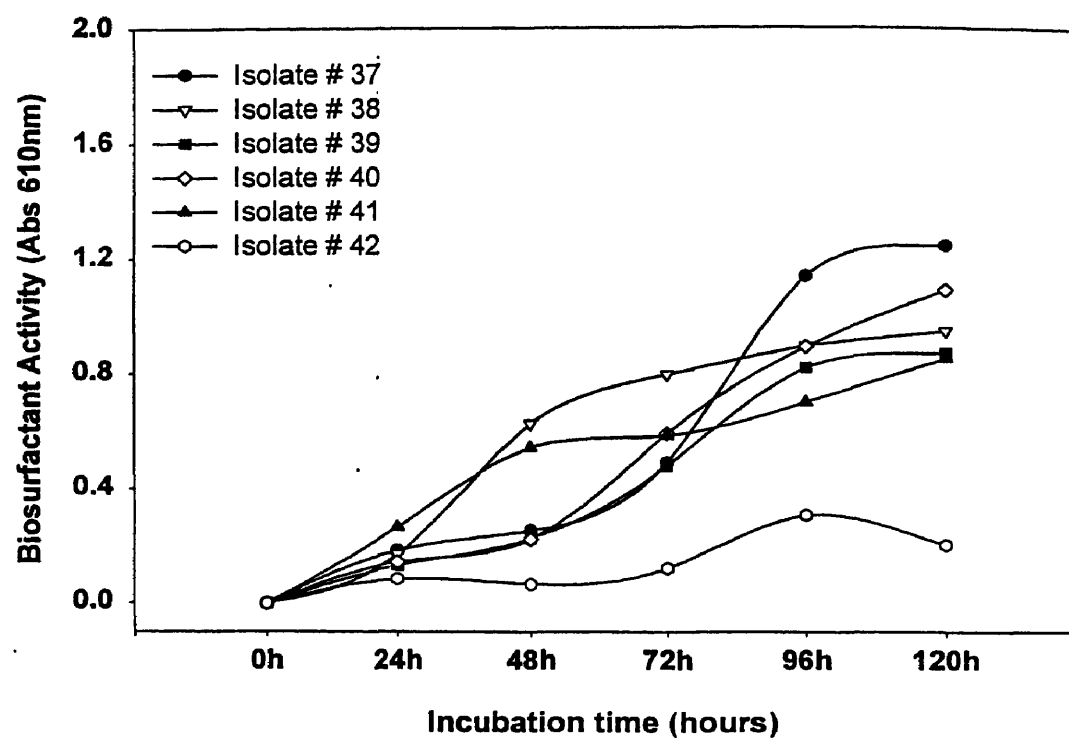


Figure 137. Biosurfactant production by Konkan isolates # 37-42

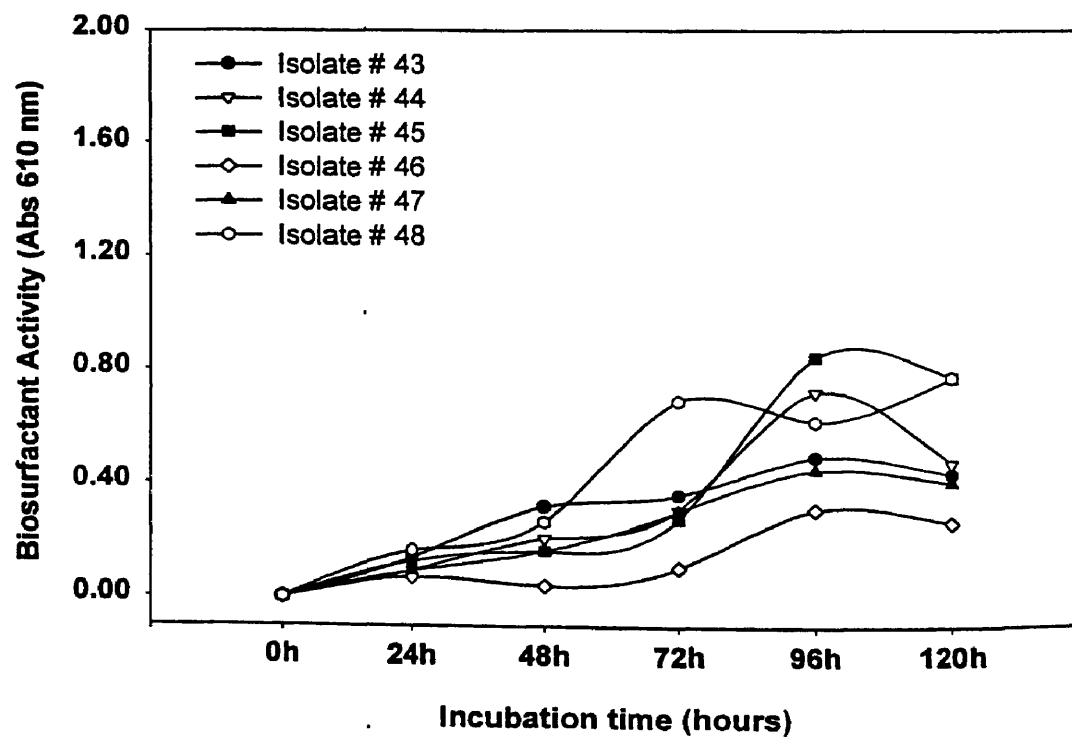


Figure 138. Biosurfactant production by Konkan isolates # 43-48

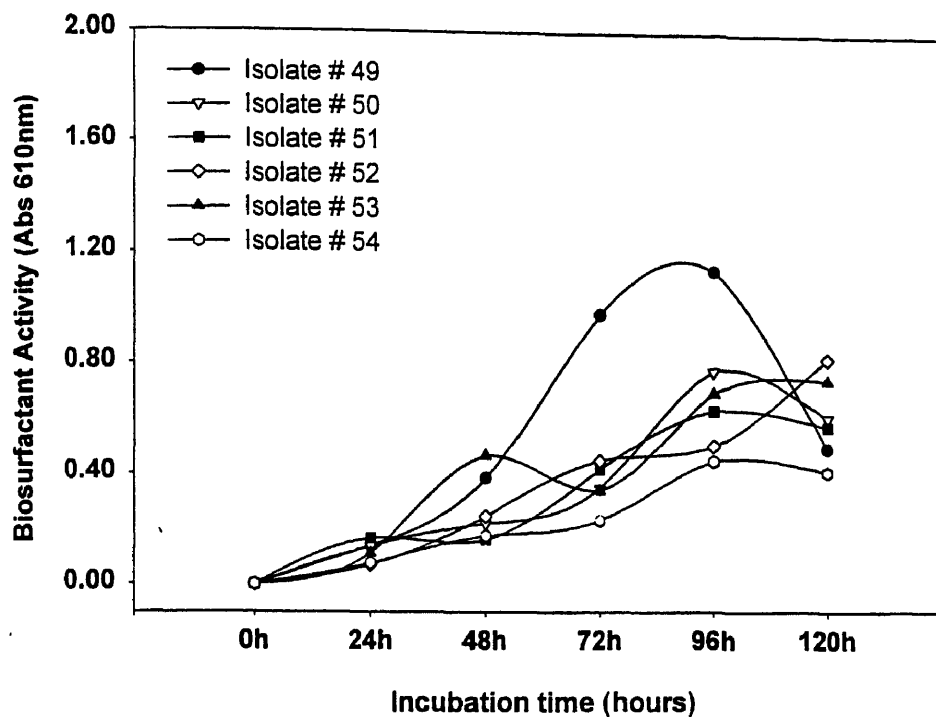


Figure 139. Biosurfactant production by Konkan isolates # 49-54

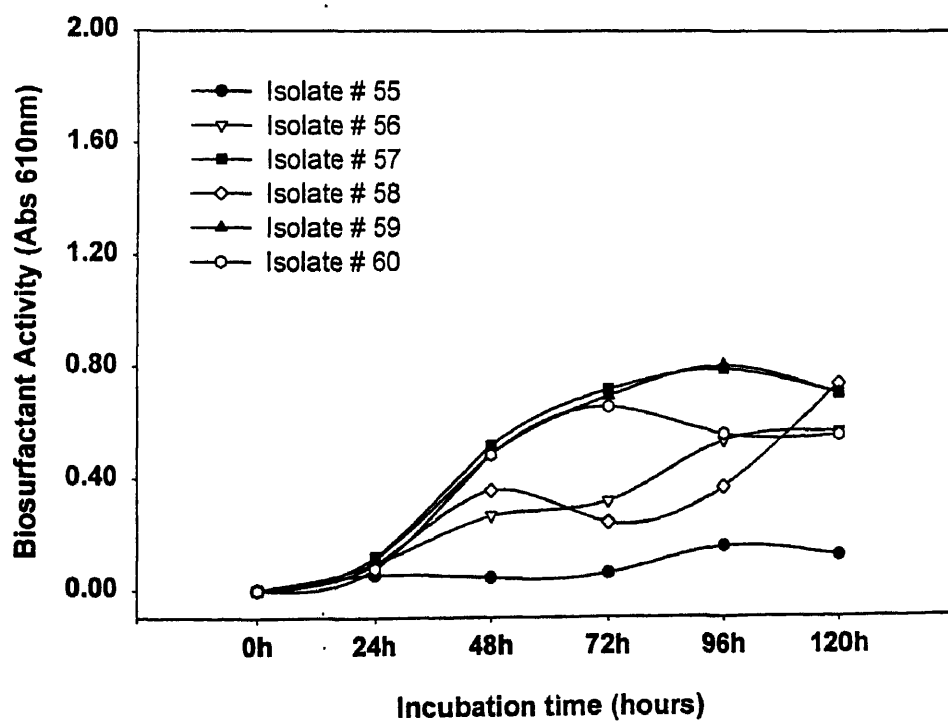


Figure 140. Biosurfactant production by Konkan isolates # 55-60

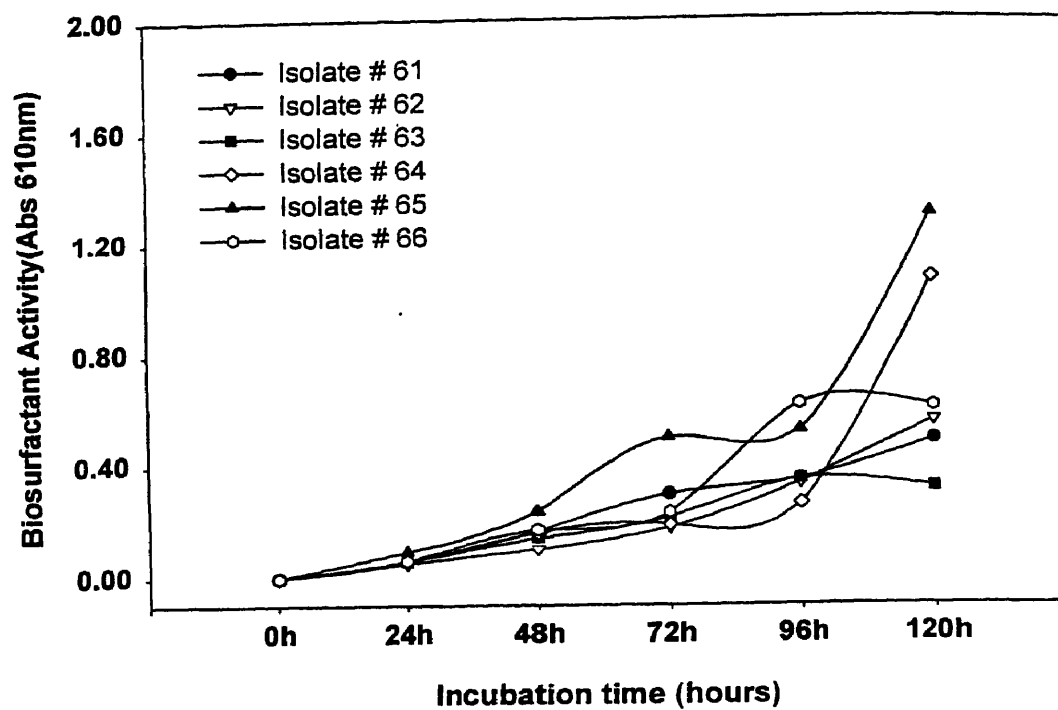


Figure 141. Biosurfactant production by Konkan isolates # 61-66

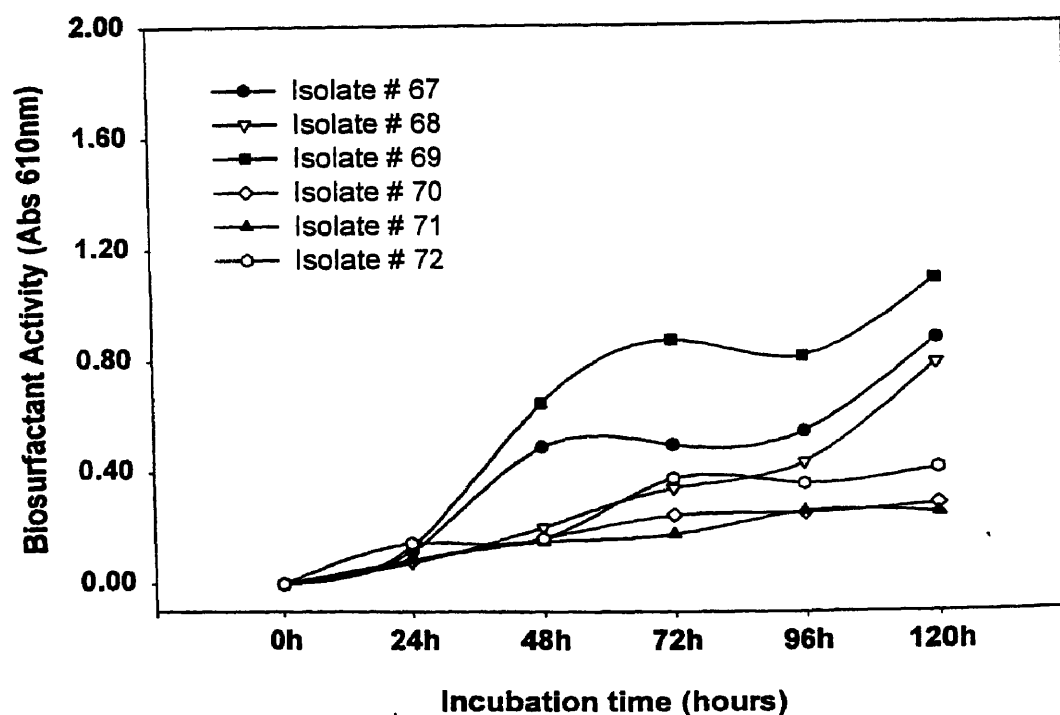


Figure 142. Biosurfactant production by Konkan isolates # 67-72

A total of 419 strains isolated from Manikaran, Mehsana and Konkan region were screened for the biosurfactant activity and their tolerance to 90°C. The best 23 strains were then screened again for their biosurfactant production activity and also thermostability (Figure 143). On the basis of their thermostable biosurfactant activity, four strains Mn-12, B-37, B-65 and Mn-246 were selected for further studies (Figure 144). These strains were tested for maximum growth and maximum thermotolerance of biosurfactant on various carbon and nitrogen sources.

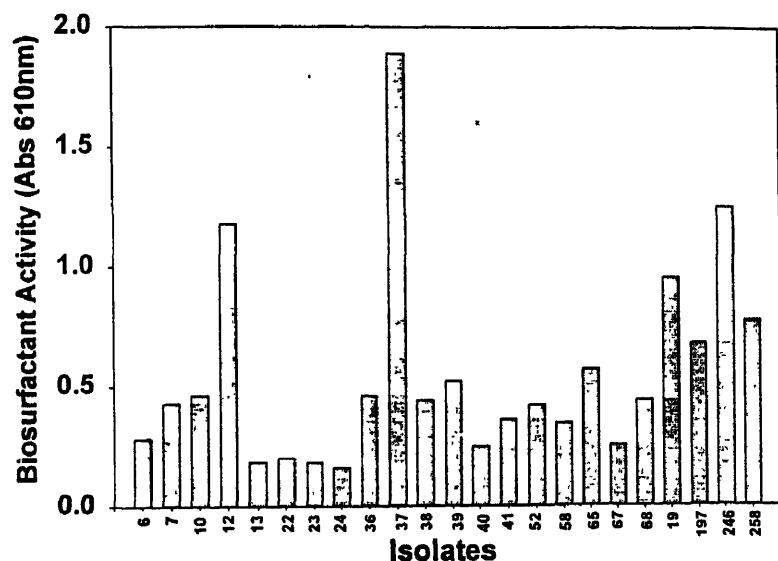


Figure 143. Biosurfactant production by the selected isolates

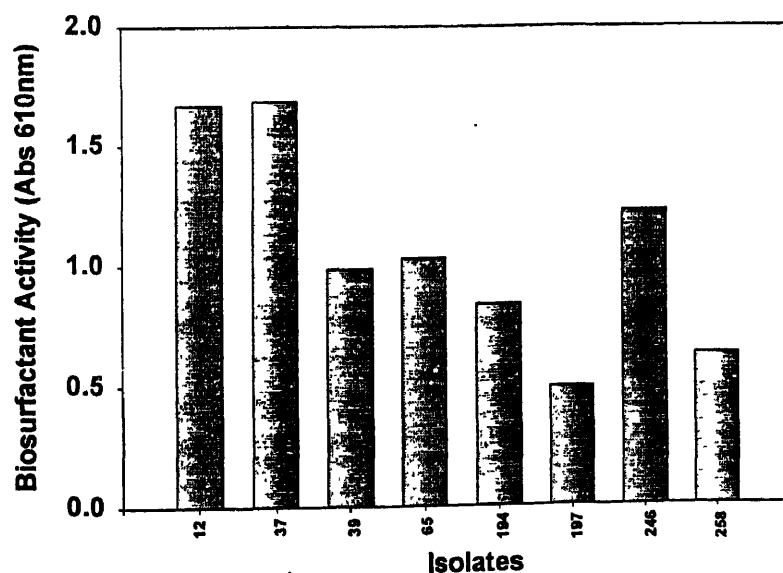


Figure 144. Biosurfactant production by the selected isolates

Optimization of culture conditions for maximum biosurfactant production

Growth of biosurfactant producing selected bacterial strains on various carbon and nitrogen sources were monitored in test tubes. The biomass production was visualized as maximum in the cultures grown in Luria broth (LB) over the cultures grown in glucose, mannitol, fructose, sucrose, arabinose and maltose. Also, minimum emulsification of crude oil in culture broth was seen in all these sugars. However, in LB good emulsification seen turning the whole culture black with micelles of crude oil.

Similar results were obtained wherein the studies were carried out for standardization of nitrogen source. Though ammonium nitrate produced better biomass over ammonium chloride, sodium nitrate and urea, Luria broth produced maximum biomass and emulsification. Hence, Luria broth was used for all the future experiments.

Studies on optimization of temperature for growth and biosurfactant production resulted that growing the culture at 55°C was more favourable over growing at 70°C. However, mesophilic temperatures resulted in very poor growth of the isolates tested (Figures 145 to 164).

Generation of hyper biosurfactant producing bacterial isolates

Upon exposure to UV-254nm radiation to 3 min 10% survival of the bacterium was observed as shown in the Table 10. However, it was observed, after inducing mutation, the culture were over growing on agar plates within 13 hours of incubation thus mixing up of the isolates on the plate (Table 11). Screening of these mutants resulted in such strains, where the mutation was not stable. Hence, it is suggested to use wild strain for production of biosurfactant that was consistent producer of biosurfactant.

Table 10 Calculation of time required to induce mutation

Period of exposure (min)	0'	1'	2'	3'	5'	8'	10'
Survival of microbes	73-77	54-58	41-47	7-8	2-3	Nil	Nil

Table 11 Growth pattern of mutated cultures exposed to UV-254nm for 3 minutes

Dilutions used				
10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Mixed	Mixed	6-8	No colonies	No colonies

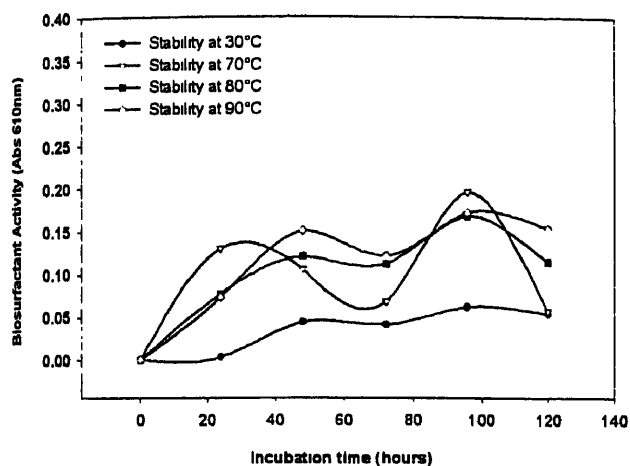


Figure 145. Biosurfactant production by Mn in 10% LB grown at 55°C

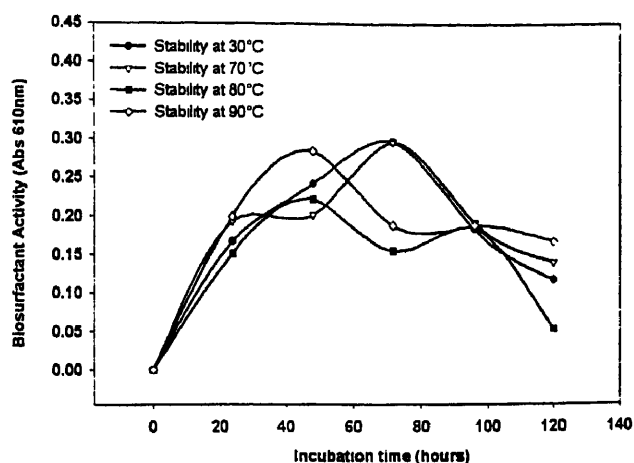


Figure 146. Biosurfactant production by Mn in 100% LB grown at 55°C

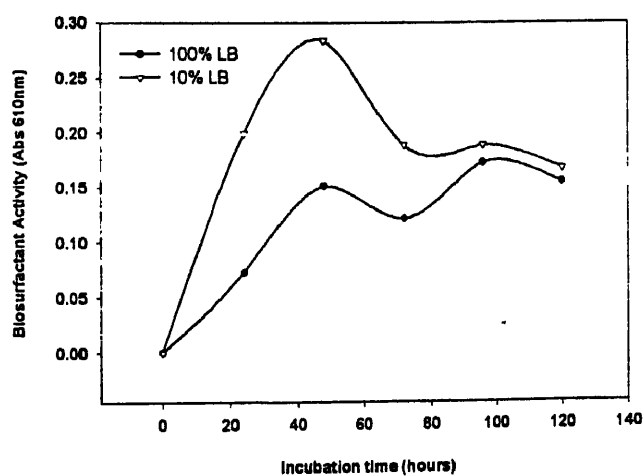


Figure 147. Effect of medium composition on biosurfactant production by Mn-4 grown at 55 °C

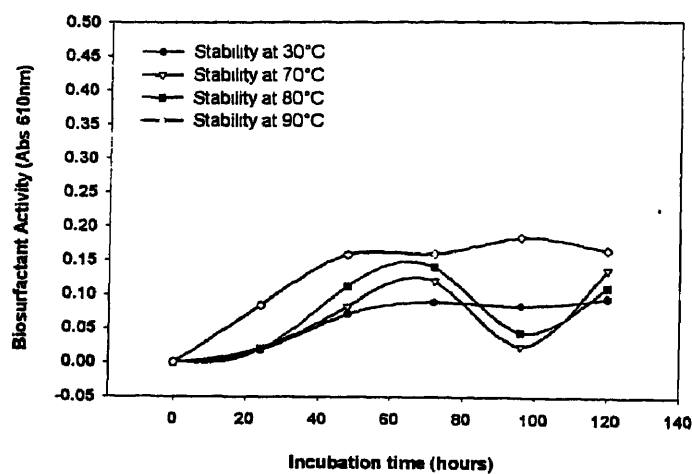


Figure 148. Biosurfactant production by Ah-32 in 10% LB grown at 55°C

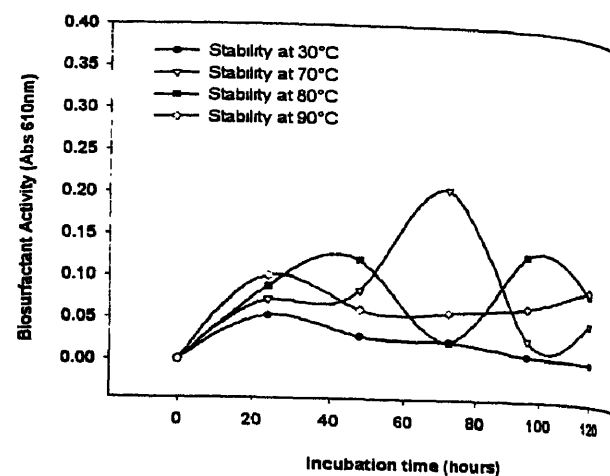


Figure 149. Biosurfactant production by Ah-32 in 100% LB grown at 55°C

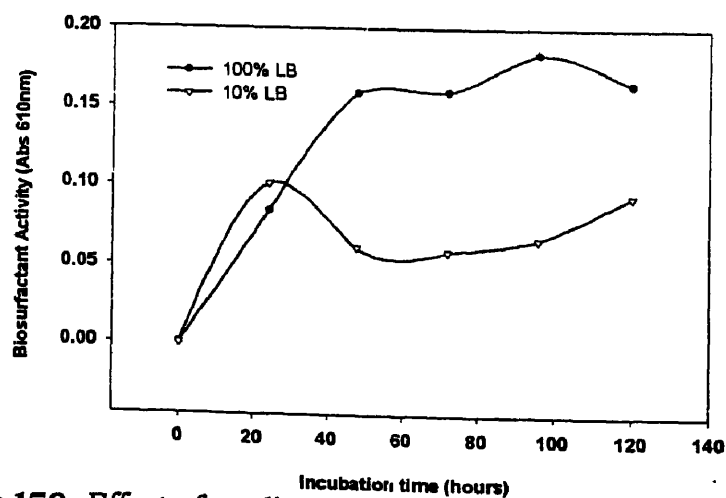


Figure 150. Effect of medium composition on biosurfactant production by Ah-32 grown at 55 °C

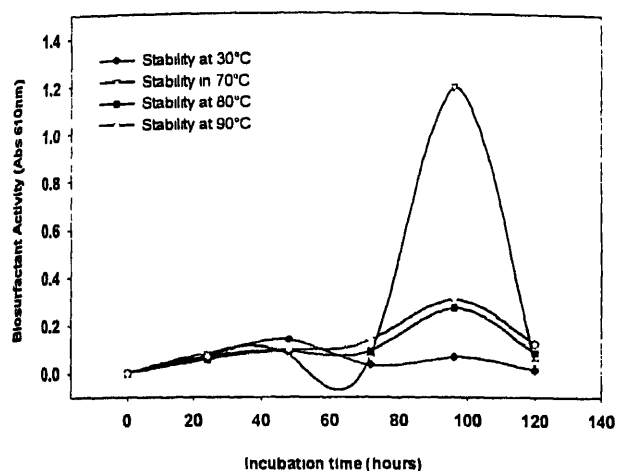


Figure 151. Biosurfactant production by Ah-91 in 10% LB grown at 55°C

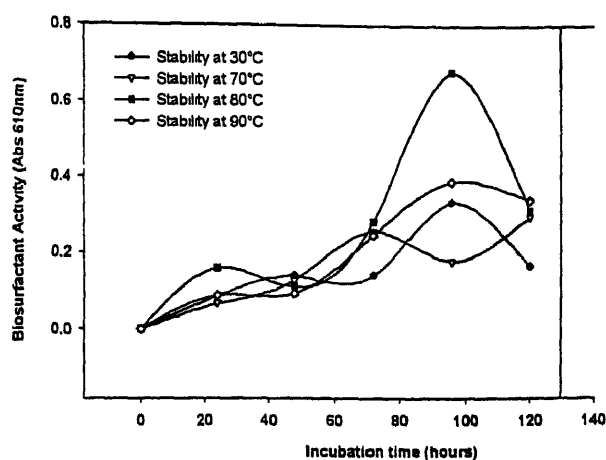


Figure 152. Biosurfactant production by Ah-91 in 100% LB grown at 55°C

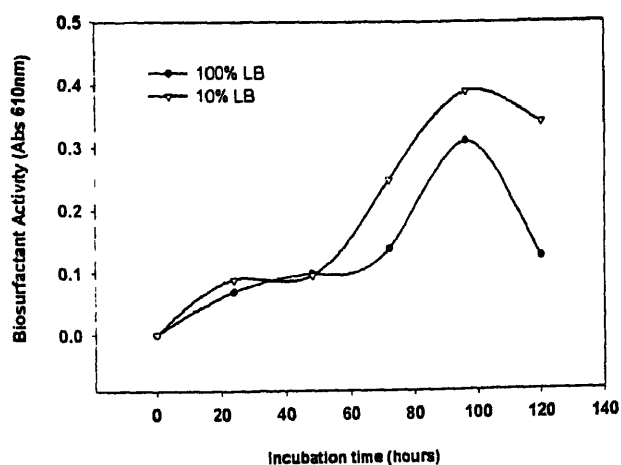


Figure 153. Effect of medium composition on biosurfactant production by Ah-91 grown at 55 °C

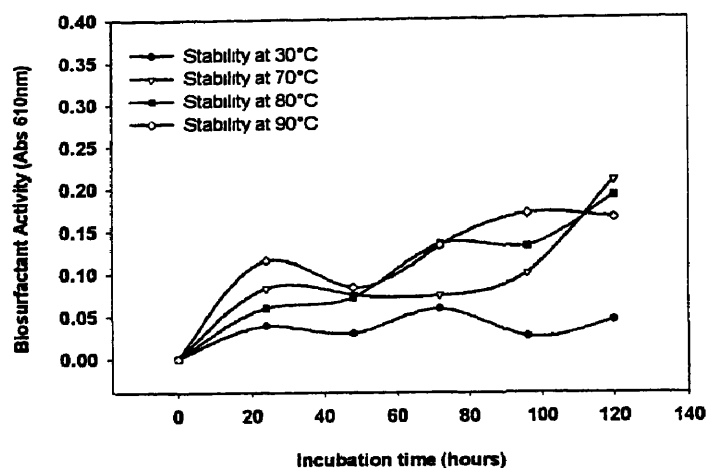


Figure 154. Biosurfactant production by Ah-102 in 10% LB grown in 55°C

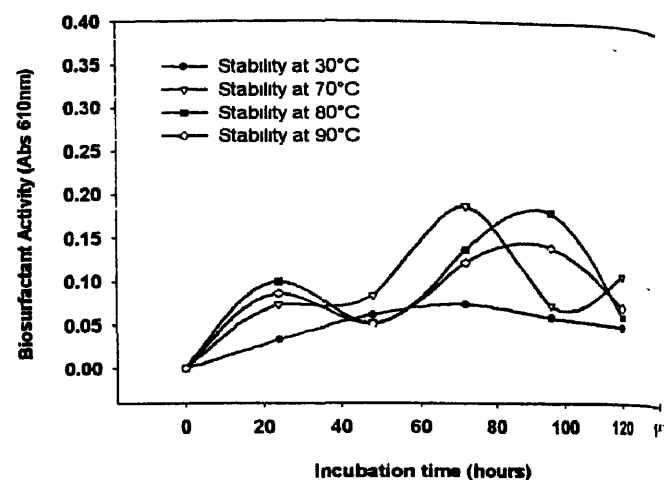


Figure 155. Biosurfactant production by Ah-102 in 100% LB grown at 55°C

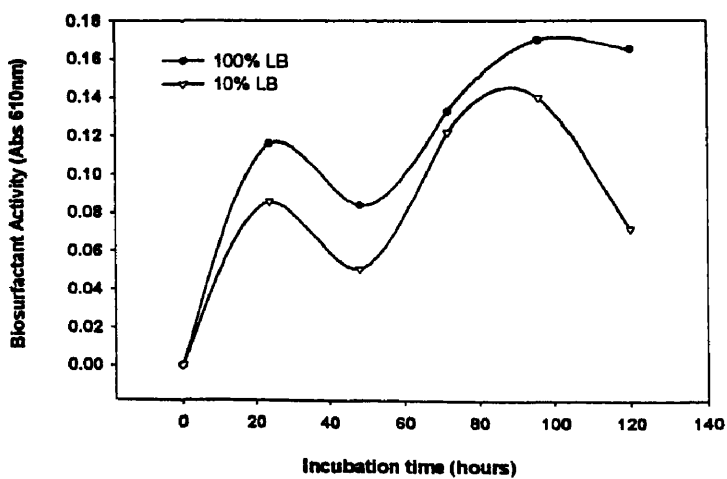


Figure 156. Effect of medium composition on biosurfactant production by Ah-102 grown at 55 °C

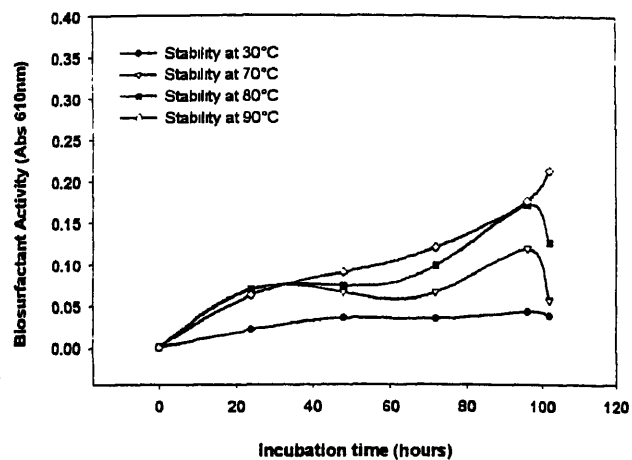


Figure 157. Biosurfactant production by Ah-109 in 10% LB grown at 55°C

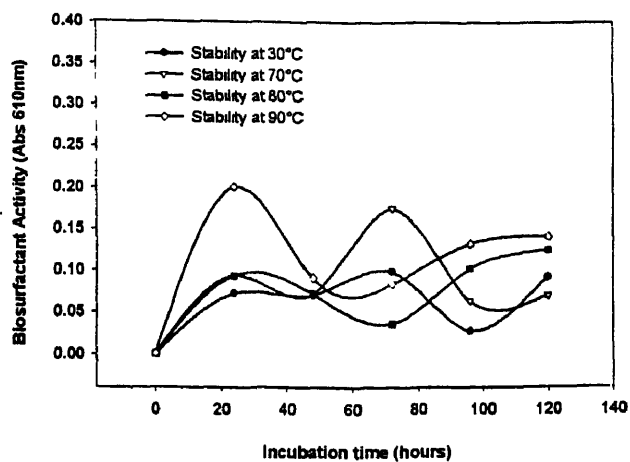


Figure 158. Biosurfactant production by Ah-109 in 100% LB grown at 55°C

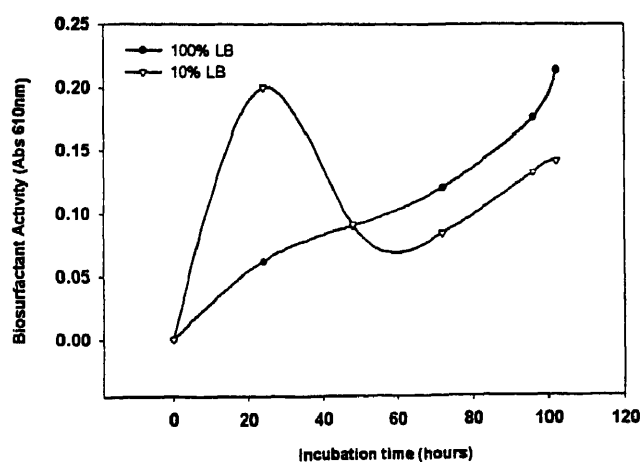


Figure 159. Effect of medium composition on biosurfactant production by Ah-109 grown at 55 °C

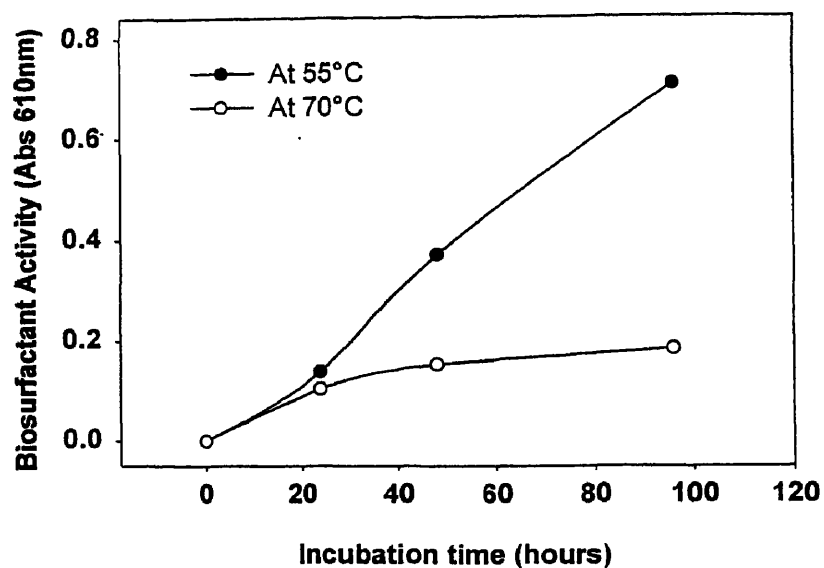


Figure 160. Effect of temperature on biosurfactant production by Mn-4 consortium

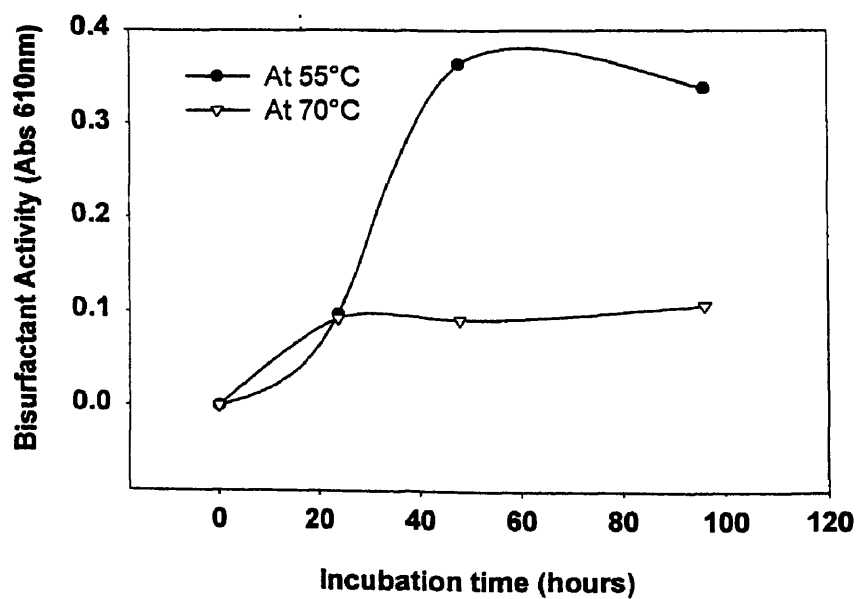


Figure 161. Effect of temperature on biosurfactant production by Ah-32 consortium

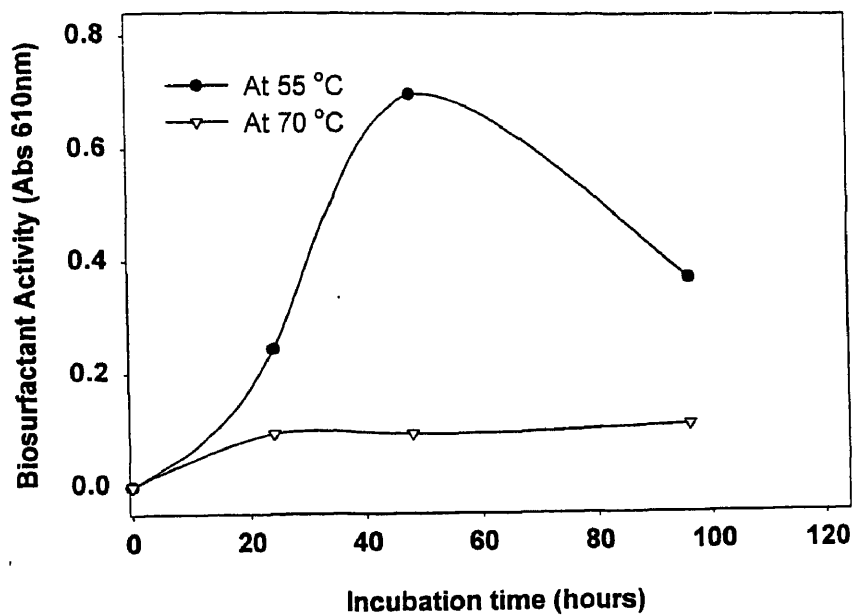


Figure 162. Effect of temperature on biosurfactant production by Ah-91 consortium

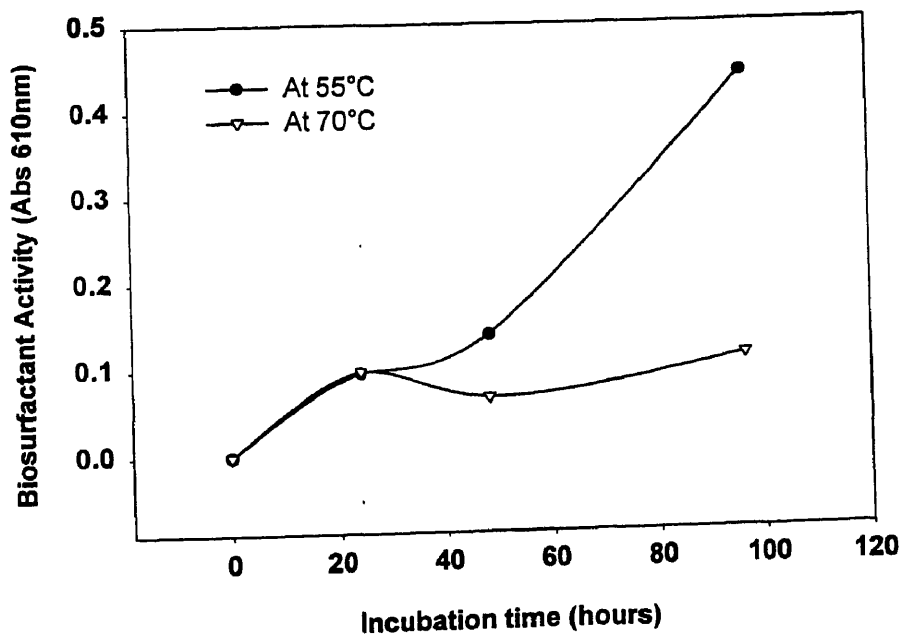


Figure 163. Effect of temperature on biosurfactant production by Ah-102 consortium

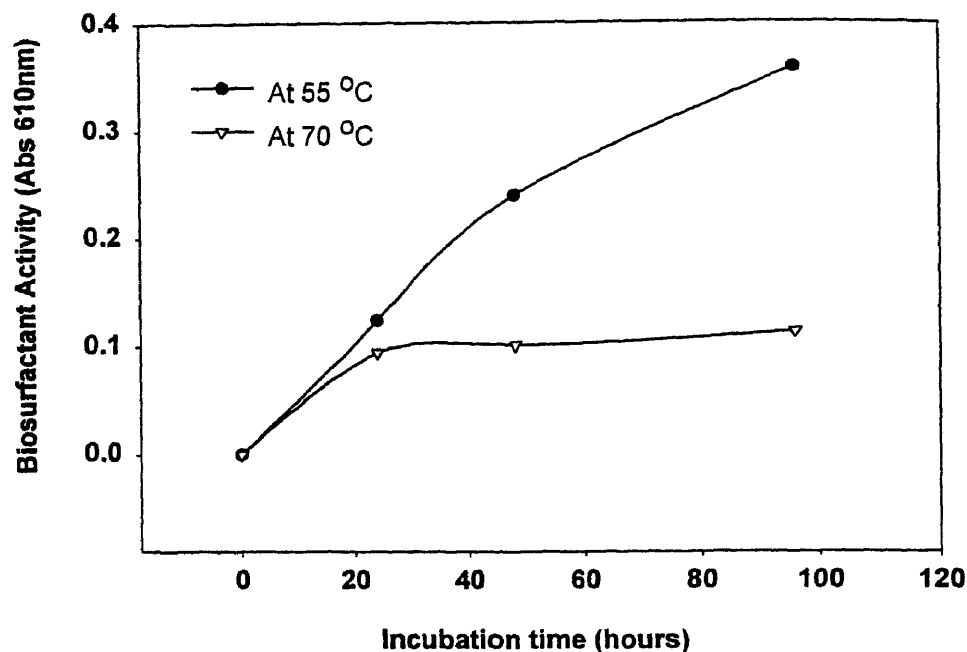


Figure 164. Effect of temperature on biosurfactant production by Ah-109 consortium

Genetic studies with the hyper biosurfactant producing isolates

Genetic studies with plasmid transformed into non-biosurfactant producer *E. coli* has shown that the receipt organism did not produce biosurfactant upon induction with 1% crude oil. This observation suggests that the biosurfactant producing genes are not present on the plasmid. It may be present in the bacterial chromosome itself.

Determination of critical micelle concentration (CMC)

Culture No. K #12 was efficiently emulsifying crude oil turning the whole culture black. Hence, this culture was selected for analysis of surfactant activity as mentioned under methodology. The biosurfactant produced by Konkan #12 lowered the surfactant activity of water to 55 dynes/cm. The culture was stored and transported in ice before analysis was carried out. CMC value was found to be around 43.0 % of the crude biosurfactant (Figure 165).

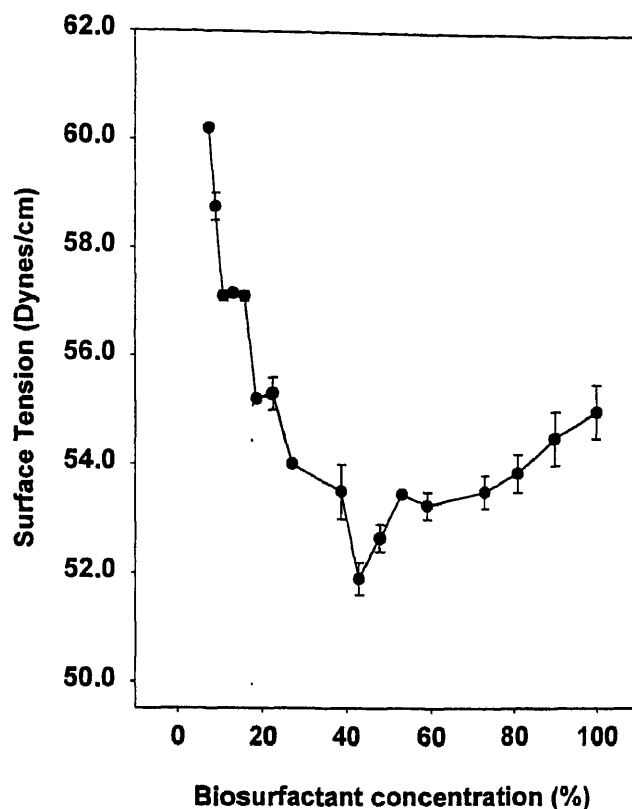


Figure 165. Calculation of Critical Micelle Concentration in biosurfactant produced by Konkan #12

Biochemical characterization of the biosurfactant

Various biochemical properties of the biosurfactant were studied after acetone precipitation of the biosurfactant from the supernatant of the cell free growth medium. The biochemical analysis of the acetone-precipitated biosurfactant is shown in Table 12. The biosurfactant of all the four isolates revealed that they were glycoproteins with very low lipid content. The protein content varied from 33 to 47 %, the carbohydrate from 43 to 58 %, while the lipid content varied from 2 to 10 %.

The properties of the biosurfactant are shown in Table 13. Among the four isolates, the biosurfactant produced by the isolate 37 and 246, reduced the surface tension to around 30 dynes/cm² while the biosurfactant of isolates 12 and 65 reduced surface tension between 40-45 dynes/cm². The biosurfactant produced by the various isolates was tolerant to 3-4% NaCl as well to pH between 6 to 9.

Table 12 Biochemical characteristics of the acetone precipitated biosurfactant

Isolate #	Constituents (%)		
	Protein	Carbohydrate	Lipid
12	33	58	8
37	47	43	10
65	40	58	2
246	39	54	4

Table 13 Properties of the biosurfactant

Properties	Culture #			
	246	37	12	65
Surface Tension (dynes/cm ²)	30-32	29-30	45-52	40-42
Yield (g/L)	1.35	1.56	1.32	1.12
Tolerance to				
NaCl	3	4	3	3
PH	5-10	6-10	5-9	5-9

Sand packed column MEOR trial with selected biosurfactants

The apparatus for the simulated sand packed column MEOR experiment by huff and puff technique (as discussed in Methodology) was used for MEOR trial with the biosurfactant and the results are shown in Figure 166.

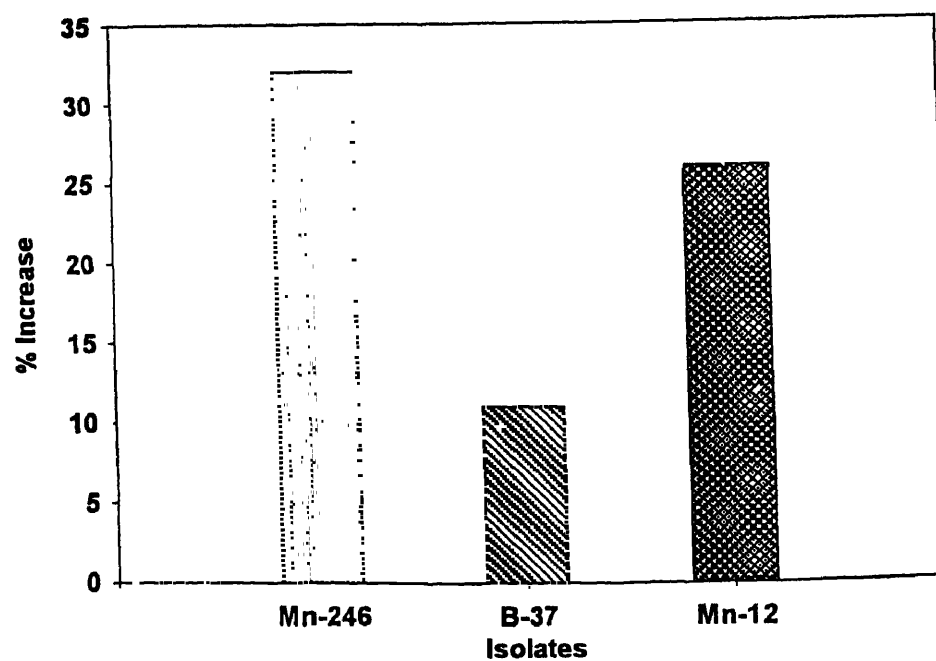


Figure 166. Enhanced oil recovery by different biosurfactants at 90 °C during sand packed column trial

The assembly was placed in a water bath maintained at 90°C. The SS assembly was filled with sand (100-200 mesh), saturated with water and then Mehsana crude oil was added at 90°C. After two days of equilibration, water was pumped from the inlet point and the water & oil coming out from the outlet was collected. The crude oil was extracted and measured gravimetrically. Subsequently the sand packed column was inoculated with the biosurfactant at 90°C. After one day of incubation at 90°C with biosurfactant, the column was pumped with water at 90°C, the water and oil coming out of the column was collected, and crude oil extracted and then measured gravimetrically.

Among the three types of biosurfactants tested, viz., Mn-246, B-37 and Mn-12, highest EOR was obtained with Mn-246 (32%) followed by Mn-12 (26%) and B-37 (12%).

Preservation and maintenance of aerobic biosurfactant producing consortium/isolate

Glycerol solution (50%) was prepared in water and sterile the glycerol by autoclaving. Now take 1 ml of active culture in pre-sterile cryotube and add 1 ml autoclaved glycerol (50%) solution aseptically. Now, the cryotubes (containing 1 ml culture and 1 ml glycerol 50% glycerol) were kept at -70°C

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Appendix

Culture media

A. Enumeration medium for anaerobic acidogenic bacteria (Braun et al., 1979)

Components	g/l
NH ₄ Cl	1.0
Yeast extract	2.0
NaHCO ₃	10.0
Glucose	1.0
MgSO ₄ ·7H ₂ O	0.1
KH ₂ PO ₄	1.2
Potassium phosphate buffer pH 7.0	5 ml
Resazurin (0.1% w/v)	1 ml
Vitamin solution	20 ml
Mineral solution	20 ml
Cysteine-HCl	0.5
Na ₂ S·9H ₂ O	0.25
Bromocresol green	0.1 g
H ₂ : CO ₂	67: 33
pH	7.8

Vitamin solution

	mg/1000 ml
Biotin	2.0
Folic acid	2.0
Vitamin B ₁₂	0.1
Pyridoxine HCl	10.0
Thiamin	5.0
Riboflavin	5.0
Nicotinic acid	5.0
DL-calcium pantothenate	5.0
Lipoic acid	5.0
p-amino benzoic acid	5.0

Trace elements

	mg/100 ml
Nitrilotriacetic acid (Sodium salt)	450
FeCl ₂ .4H ₂ O	40
MnCl ₂ .4H ₂ O	10
CaCl ₂ .2H ₂ O	3
CoCl ₂ .6H ₂ O	17
ZnCl ₂	10
H ₃ BO ₃	2
Na ₂ MoO ₄ .2H ₂ O	1

B. Isolation medium for acetogenic bacteria Balch et al. (1977)

Components	g/l
NH ₄ Cl	1.0
MgSO ₄ .7H ₂ O	0.1
K ₂ HPO ₄	0.4
KH ₂ PO ₄	0.4
Resazurin (0.1%)	0.0001 (1 ml)
Cysteine-HCl	0.5
Na ₂ S.9H ₂ O	0.5
NaHCO ₃	2.0
Bromothymol blue	1.0 ml
Vitamin solution	10 ml
Trace mineral solution	10 ml
Glucose or Molasses	5.0
pH	8.3

Vitamin solution

	mg/l
Biotin	2.0
Folic acid	2.0
Pyridoxine HCL	10.0
Thiamin-HCL	5.0
Riboflavin	5.0
Nicotinic acid	5.0
DL-Calcium pantothenate	5.0
p-Aminobenzoic acid	5.0
Lipoic acid	5.0
Vitamin B ₁₂	0.1

Trace mineral solution

	g/l
Nitrilotriacetic acid (sodium salt)	1.5
MgSO ₄ .7H ₂ O	3.0
MnSO ₄ .2H ₂ O	0.5
NaCl	1.0
FeSO ₄ .7H ₂ O	0.1
CoCl ₂ /CoSO ₄	0.1
CaCl ₂ .2H ₂ O	0.1
ZnSO ₄	0.1
CuSO ₄ .5H ₂ O	0.01
Alk (SO ₄) ₂	0.01
H ₃ BO ₃	0.01
Na ₂ MoO ₄ .2H ₂ O	0.01
pH	7.0

C. Basal mineral medium

Components	g/l
K ₂ HPO ₄	1.0
KH ₂ PO ₄	1.0
MgSO ₄ . 7H ₂ O	0.5
NH ₄ Cl	0.5
Trace mineral solution	10.0 ml
Vitamins solution	1.0 ml

Trace Mineral solution

	g/l
Nitrilotriacetic acid	1.50
MnSO ₄ . 2H ₂ O	5.00
FeSO ₄	0.01
CaCl ₂	0.10
AlK(SO ₄) ₂	0.01
H ₃ BO ₄	0.10
Na ₂ MoO ₄	0.01

Vitamins solution

	g/l
Biotin	0.002
Folic acid	0.002
Pyridoxine HCl	0.010
Riboflavin	0.005

Thiamine HCl	0.005
Nicotinic acid	0.005
DL-calcium pantothenate	0.005
Vitamin B ₁₂	0.001
PABA	0.005
Lipoic acid	0.005

D. Luria Broth

	g/l
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0

Reagents

A. Biuret reagent

	g/l
NaOH	300.0
CuSO ₄	1.2

Sodium hydroxide was dissolved in ice-bath and cuprous sulphate was added slowly with continuous stirring.

B. Bradford reagent

Coomassie Brilliant Blue G-250	100.0 mg
Phosphoric acid (v/v)	85%

Coomassie brilliant blue was dissolved in 50 ml of 95% (v/v) ethanol and 100 ml of 85% (v/v) phosphoric acid was added to the solution. The volume was made to 1000 ml and the solution was filtered till blue color appeared. The reagent was stored in an amber colored bottle.

C. 50 mM phosphate buffer

Dissolved 14.196 g Na₂HPO₄ and 15.601 g NaH₂PO₄ each in 500 ml distilled water separately to prepare stocks of 200-mM solutions. Now for preparing 50 mM of working buffer 39 ml of NaH₂PO₄ (200 mM solution) and 61 ml of Na₂HPO₄ (200 mM solution) are mixed and the volume is made to 400 ml with distilled water.

D. Fixer

In 1 litre of 200 mM phosphate buffer, 80 g of *para*-formaldehyde (60°C) and 80 ml of Glutaraldehyde (25%) is added and the volume is made to 2 litre with distilled water.

E. Glycerol stock (50%, v/v) for anaerobic culture maintenance

In 100 ml of concentrated glycerol, 100 ml distilled water is added along with 0.2 ml of resazurin solution (0.1% w/v) as an anaerobic indicator in the anaerobic bottle (500 ml capacity). The contents are heated to 60 to 70°C and oxygen-free N₂ is passed to make the contents anaerobic. The contents are cooled to room temperature under continuous N₂ passing and reducing agents (Cysteine-HCl and Na₂S.9H₂O) are added @ 0.05% (w/v) each. The bottle is crimped under anaerobic conditions and autoclaved at 121°C for 15 minutes.

F. Glycerol stock (50%, v/v) for aerobic culture maintenance

In 100 ml of concentrated glycerol, 100 ml distilled water is added in the 500 ml capacity screw-capped flask. The contents are autoclaved at 121°C for 15 minutes.

G. Anthrone reagent

A 0.2 g anthrone is dissolved in 100 ml concentrated sulphuric acid in screw-capped flask and chilled for two hours in ice before use.